

Making ends meet in South Africa

As it struggles to overcome the legacy of apartheid, South Africa needs to protect and strengthen the most productive parts of its science base. That requires a tough approach to the redistribution of resources — and determination to tackle big spenders.

WITH the immediate euphoria of the ending of apartheid receding rapidly, South Africa is struggling with the daunting task of forging a modern industrial economy able both to meet the needs of all its people, and ensure economic success in the global market-place. A critical component of these is the restructuring of its science base to ensure that both goals are pursued as effectively as possible. In doing so, South Africa needs to redress the idiosyncrasies of its past allocation of research funding. These were based on the priorities of a besieged nation, pre-occupied with maintaining apartheid and the privileges it conferred on its white minority. Reform in this sphere, as in so many others, is long overdue.

Significant progress is represented, at least in principle, by the white paper that was published in September, and which will soon come into effect (see page 11). The paper sets out some useful proposals for reorganizing the way that research funds are distributed between different sectors, and linking research to economic and social needs. Disappointingly, it fails to provide incentives to the private sector to invest more in research.

One reason being given for not offering tax concessions, either for increased R&D spending or for donations to museums by either companies or individuals, is that the revenue services lack the capacity to administer them. But that is a weak excuse, given the potential benefits at stake and the relatively low cost of employing a few skilled accountants.

There is also much sound sense in the recent report of the National Commission on Higher Education, which sets out a framework for the future financing of tertiary education. The government's response is due early next year. In addition to accepting radical proposals on student funding, it needs to spell out clearly how the research component of university funding is to be financed. At present, fifteen per cent of federal support for universities is allocated for research using a formula based on publications in accredited journals. But one result is that this strongly favours the five major universities, in which almost two-thirds of South Africa's research is carried out. The government should maintain these existing centres of productivity. But, in return, it should demand that they continue steadily to transform themselves into institutions that are broadly representative of South African society — or at least the region which they serve.

Another critical issue is how limited research funds should be distributed more effectively among the seven research councils (which carry out significant proportions of their research in-house), the proposed National Research Foundation (which will distribute funds to the universities, polytechnics and museums), and the new National Innovation Fund. Closing down or privatizing large sections of some of the research councils may be essential if they are unproductive, or perform consultancy work which does not merit public subsidy by the taxpayer. And it would be a mistake if both the military research budget and that of the Atomic Energy Corporation — the two greatest dinosaurs of the apartheid era in terms of research spending — were to escape the knife.

Dismantling outdated structures can be as difficult as creating new ones. The new minister, Lionel Mtshali, has the oppor-

tunity to make his mark, but will need political backing to do so. If the government has the courage to implement the requisite changes, 1998 could turn out to be the year of reckoning and of exciting opportunity for South African science. □

Peer review and the courts

Despite an out-of-court settlement last week, science must not rely on litigation to keep its house in order.

A TWELVE-YEAR battle between Immunex and Cistrion has ended with the payment by the former to the latter of \$21 million, as well as patent rights to the interleukin-1 protein at the centre of their dispute (see page 4). Cistrion sued Immunex because it believed that a scientist at that company had misappropriated information about the protein's structure from a paper, submitted to *Nature* by a Cistrion-sponsored team, which he was asked to review. Cistrion pointed to suspicious similarities: subsequent patent applications filed by Immunex replicated errors in DNA sequences contained in the original.

If the case had come to court, it would have called for a potentially significant ruling on the untested question of whether the confidentiality of the peer review process is legally binding, and on the extent to which a reviewer is legally (as well as ethically) constrained in his or her use of that material. This notion of confidentiality underpins the entire peer review system. Whether the law of the United States underpins that notion is a question which for now must, after all, remain unanswered.

Cistrion would have argued that the confidential nature of the reviewing task is not only essential in the conduct of science, but also well understood by the scientific community, through custom and practice. Immunex's lawyers would have said that, in practice, the precise requirements of confidentiality are poorly understood — and indeed are only pursued at the reviewer's discretion.

The decision by Immunex to pay \$21 million to Cistrion, rather than take its argument to court, is welcome. Though considerably less than Cistrion asked for, it is a useful amount of compensation for the loss of information on a protein which has turned out to be of very little commercial value. It will ensure an early and happy retirement for each of Cistrion's seven employees, should they suddenly tire of molecular biology. More importantly, it will send a clear signal to any scientist who may be torn between corporate zeal and professional ethics that the former practised at the expense of the latter can have a high price.

But satisfactory as it may be, the outcome of a high-visibility case like this one can have only a marginal effect in holding the line against scientific misconduct. More often than not, there are no faulty data with which to trace misbehaviour, and it is only the decency and honesty of individual researchers that hold the line, and maintain the integrity of science. In particular, much too often, researchers insist on tight confidentiality when they are authors, but are markedly less rigorous when they are sent papers to review. They too need to take note of the dangers. □



British scientists seek recognition of role in 'life on Mars' debate ...

London. The UK government last week joined the debate over whether life exists — or has existed — on Mars by hosting a conference in London on extra-planetary life, a major aim of which was to show that British research does not lag behind that of the United States.

Indeed, a press release issued prior to the meeting, eagerly taken up by newspapers on both sides of the Atlantic, said that research carried out by British scientists "offered the strongest support yet for the hypothesis that life once existed on the planet Mars".

The meeting brought together most, if not all, of the UK planetary community. It was organized jointly by Ian Taylor, Britain's science minister and a keen enthusiast for space research, and Colin Pillinger, professor of astronomy at the Open University, and an acknowledged authority on carbon in extraterrestrial rocks.

Both say they are concerned that relevant British space research — and Pillinger's work in particular — had been unfairly ignored in the publicity that surrounded announcement by the National Aeronautics and Space Administration (NASA) in August of evidence of microbial activity in a martian meteorite ALH84001 (see box, right). At Taylor's request, the two met at Pillinger's laboratory in Milton Keynes just days after the announcement, and resolved to take steps to remedy the situation.

Seven years ago Pillinger and colleagues reported the discovery of carbon compounds in a martian meteorite, EETA 79001 (see *Nature* 340, 220; 1989). At the time, Ian Wright, one of Pillinger's co-authors, gave several interviews in which he explicitly discounted the possibility that the paper provided evidence of life on Mars.

But Pillinger says this work was an important signpost and helped pave the way for NASA's announcement and the subsequent publication of conclusions by researchers that are being interpreted as the first signs of life on the red planet. His work was not explicitly cited by the NASA researchers.

Taylor says that, when President Clinton announced plans for a US space science summit in the wake of the 'life on Mars' announcement, he was concerned that British researchers should not be left behind. "I genuinely felt that the excellent work done in the UK had been underestimated in all the publicity and hype at the time," he says. "British science should be given its proper platform."

Paul Murdin, director of science at the

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Pause for thought: Colin Pillinger, of Britain's Open University, who announced new findings last week.

British National Space Centre, describes last week's meeting as a "stunning" achievement and says Britain's planetary science research potential has been clearly underestimated and should be more focused. Indeed, Murdin, who is also head of astronomy at the Particle Physics and Astronomy

Research Council, says he would "take seriously" any proposal to establish a single UK centre for Solar System sciences intended to "harness the terrific wealth of talent".

Taylor says he would "welcome any such proposal", particularly if it involved a "first-rank university or a number of universities".

The meeting, held at the Royal Society, heard presentations from more than 20 British scientists engaged in planetary science and related activities, including astronomers, geologists, and members of life sciences communities.

Although the meeting itself was strictly scientific, several journalists had been invited to help publicize its conclusions. Pillinger, together with his colleague Ian Wright, presented unpublished results of a further analysis of the rock that featured in their 1989 *Nature* paper.

Their results appear to confirm that the organic carbon compounds discovered in ►

...as NASA's claims win few converts

Washington. Despite the announcement last week by a group British researchers that they had observed signs of biology in a second martian meteorite (see above), most space scientists and biologists remain sceptical that the claim made this summer by a US team of past life on Mars will stand up to scrutiny.

Although details of the new work have yet to be published, the scientific community has had three months to examine the evidence of ancient Martian life proposed last August by David McKay and colleagues at the National Aeronautics and Space Administration (NASA) Johnson Space Center in Houston and Stanford University. Few scientists have been won over by their arguments, and several have proposed alternative explanations for the data.

But no obvious flaws have yet been found in the work, says Timothy Swindle of the University of Arizona, who summarized dissenting viewpoints during a panel discussion of the Mars finding at a recent meeting of the American Astronomical Society's Division of Planetary Sciences (DPS). "If I had to bet, I'd bet that [McKay *et al.*] are not right, but I don't think that's been demonstrated," he says, adding that an informal canvas

of his colleagues gave the NASA team a chance of between ten and fifty per cent of being proved correct.

Swindle's presentation at the DPS meeting — and a similar 'rebuttal' led by Harry McSween of the University of Tennessee at a recent meeting of the Geological Society of America — focused on several key questions regarding the McKay group's claims. One concerns temperature.

McSween and others argue that the carbonate 'globules' containing the purported fossils actually formed at temperatures too high for microbes to exist, which would virtually rule out the martian life hypothesis (see *Nature* 382, 49–51; 1996). Electron micrographs of the 'fossils' themselves are also under suspicion, says McSween; some microscopy experts believe the structures are simply artefacts of a coating process which was used to prepare the samples.

Jeffrey Bada of the Scripps Institution of Oceanography in San Diego believes the McKay group is being fooled by terrestrial contamination. He and his colleagues have also found biogenic amino acids and polycyclic aromatic hydrocarbons (PAHs) in the martian rock that was examined by the British ►

► the relatively young martian meteorite EETA 79001 seven years ago were of martian rather than terrestrial origin. One journalist was given an exclusive interview with Pillinger and the following day's headline read: 'Life on Mars was found by Britons'.

Pillinger also decided to publicize separate results, obtained just one week before the meeting, of an isotope analysis of small amounts of carbonate taken from the much older meteorite ALH 84001. These results appeared to corroborate the findings of the NASA researchers by suggesting that the carbonates were formed from microbially-produced methane.

Pillinger says the researchers do not regret discussing unpublished research directly with the media, even though he was aware that this might reduce its chances of being published by some journals. He says such an action was necessary for the meeting to achieve one of its main objectives — to generate publicity for British science.

Taylor says he disagrees with suggestions that the meeting amounted to 'science by press conference', preferring to describe Pillinger's actions as "instant peer review". The minister also rejects suggestions that such procedures encourage a 'publicize first, publish second' mentality. "I don't think scientists will depart from the desire to publish or get citations. Colin Pillinger is already well established. He quite naturally felt he could take a risk. I give him credit for that."



Monica Grady of the Natural History Museum in London agrees that scientists have a duty to communicate to the public, although she adds that she is personally not in favour of portraying science "as a competition or as a conflict".

Grady, who is the third author on the 1989 *Nature* paper, says she is less sceptical of the NASA announcement of life on Mars than she was in August, and acknowledges that she is "a great believer that we will find life in a planetary system". But she emphasizes that definitive answers to this question will be found only by further research and from future Mars missions.

Ehsan Masood

► team, EETA 79001.

Furthermore the pattern of PAH types and distributions is an "almost exact overlay" of what McKay's group reported for their meteorite, Allan Hills 84001, says Bada. But he adds that the amino acids and hydrocarbons are almost certainly from Antarctica, not Mars. Bada's detailed analysis of ice from the Allan Hills region shows that it's "loaded with [PAHs], and it looks just like what's in the meteorite".

These and other nagging doubts have led even NASA researchers — including those working on plans to revamp the agency's strategy for exploring Mars — to be extremely cautious in supporting the McKay team's conclusions. According to John Logsdon, however, of the Space Policy Institute at George Washington University, the 'discovery' has already had a significant effect at NASA.

Logsdon points out that the justification for robotic exploration of the planet (the US Mars Global Surveyor spacecraft, the first of three missions headed for Mars this year, was scheduled to launch this week) has shifted more explicitly to include a search for life. And he says that extreme cuts to NASA's budget floated in some budget projections earlier this year now seem unlikely.

Meteorite researchers also will

benefit from the finding. NASA and the National Science Foundation (NSF) are each spending about \$1 million to fund work that might settle whether the Mars rocks contain signs of life, and will ask for proposals from the scientific community later this month.

Forty-seven research teams already have samples of the twelve known martian meteorites, and 16 more have requested samples. Allocation of samples has been suspended until after the NASA/NSF solicitation, however, partly so that the committee in charge of deciding who receives samples can strengthen its expertise in certain scientific areas, including palaeobiology and organic geochemistry, according to Marilyn Lindstrom, a meteorite curator at the Johnson Space Center. Lindstrom says that samples will be allocated independently of funding decisions, and that the NSF and NASA will coordinate their research funding so as not to overlap.

Many space scientists hope the additional money and attention will draw new blood to the field and lead to interdisciplinary collaborations. McKay's team plans to continue looking for cellular structure in its microfossils and pursuing other investigations, but is keeping quiet about how and when it might release further results.

Tony Reichhardt

Settlement deprives peer review of its day in court

Washington. A Seattle-based biotechnology company has agreed to pay \$21 million to a rival which claimed that it stole information subsequently used for a patent application from a paper under confidential review for publication in *Nature*.

The out-of-court settlement, which was reached on 31 October — only days before the case was due to be heard in a Seattle courtroom — deprives the science community of a legal judgement on the confidentiality of the peer review process.

Cistrion, a small, New Jersey-based company, had taken Immunex of Seattle to court on the basis that an Immunex scientist had taken data on a protein, interleukin-1 (IL-1), from a paper submitted to *Nature* in 1984 by a Cistrion team, and that Immunex had then used it for its own research and patent applications. The paper was rejected for publication, and IL-1 turned out to have little commercial significance.

Evidence for the theft included a set of errors in a DNA sequence in the original paper, which was later repeated in Immunex's patent applications. Immunex denied stealing the data. But according to documents filed to the court, the company was preparing a defence which argued that the peer review process is not legally confidential, and that material cannot be regarded as confidential once submitted for publication in a peer-reviewed journal.

Immunex has now agreed to pay \$21 million to Cistrion over a three-year period and assign some of its patents on IL-1 to the New Jersey company. When prospects for IL-1 were brighter, Immunex received more than \$100 million from a major pharmaceutical company for a share of its work on the protein.

Cistrion initially sued for \$150 million, which it later reduced to \$70 million. A joint statement from the two companies said that the settlement "was made without any concession or admission of liability".

Henry Grausz, chairman of Cistrion — which has seven employees and had sales last year of just \$700,000 — said it "believes it holds a strong position in the IL-1 area". Immunex has 800 employees and annual sales of \$160 million. It is now fighting to regain some of the settlement costs from its liability insurers.

Had the case gone to court, the outcome could have hinged on whether material submitted to a peer-reviewed journal is legally confidential. "If it had come to court, the point would have been decided," says Sir John Maddox, former editor of *Nature*, who was due to appear as an expert witness for Cistrion.

Colin Macilwain

German physicians warn of genetics risks

Munich. The German section of International Physicians for the Prevention of Nuclear War (IPPNW) has called for a full debate in the German parliament on the Council of Europe's proposed Bioethics Convention, claiming that it is not sufficiently restrictive on experiments involving embryos and on genetic screening, and fails to provide adequate protection for the mentally handicapped.

The demand for such debate was made at a meeting in Nuremberg last week held to commemorate the 50th anniversary of the trials of doctors who experimented on concentration camp prisoners during the Nazi era. At the meeting, the IPPNW, whose parent body won the Nobel peace prize in 1985, also called for a moratorium on the development and use of new genetic screening tests until laws have been introduced protecting individuals against their possible misuse.

The meeting endorsed the text of a declaration warning that if the draft bioethics convention is approved, there is a serious risk that Germany's dark history of human experimentation will repeat itself. The declaration has already been signed by more than 14,000 individuals, including representatives from the medical, academic and political communities, as well as groups such as charities for the mentally handicapped.

The document was drawn up by Michael Wunder, a psychologist based in Hamburg who works with mentally handicapped people. He argues that the draft convention compromises the first article of the so-called Nuremberg code of 1947, which was written at the end of the doctors' trials to provide a legal basis for ethical scientific research.

The code says that the voluntary consent of the human subject in medical experiments is "essential", and also specifies that anyone taking part in such an experiment must be capable of giving consent. It was modified slightly by the 1964 Helsinki declaration by the World Organization of Physicians to permit experiments without consent on an individual if they are of potential direct benefit to the individual's health.

In contrast, the proposed bioethics convention would allow research to be carried out to determine general mechanisms of a disease suffered by a person incapable of giving consent ('legally incapacitated persons'). This would be of no direct benefit to the patient, but would benefit others afflicted with the same disease in the future. Wunder says that the convention opens a door to potential abuse which may not be easily closed.

The declaration also argues that the convention is too liberal towards embryo experimentation and genetic screening. In fact, the latest draft of the convention takes no stand

on the issue of embryo research, requiring only that the embryo 'be protected' under national law in those countries where it is undertaken. Genetic testing would be permitted for health purposes or for scientific

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Past crimes: the conduct of Nazi doctors such as Karl Brandt (above) is influencing the debate in Germany.

research linked to health purposes, accompanied by appropriate genetic counselling.

Wunder's document was initially drawn up for a meeting of the IPPNW's working group on the history of euthanasia held last June in Grafenecker, a small town in south-west Germany, where 10,000 mentally handicapped and psychologically ill people were gassed by the Nazis in 1940. Now known as the 'Grafenecker declaration' on bioethics, the declaration was endorsed at the Nuremberg meeting.

But Ludger Honnefelder, director of the Bonn Institute for Science and Ethics, and a member of the Council of Europe's expert committee responsible for the drawing up of the convention, rejects the declaration's criticisms, which he says are based on a "wilful" misunderstanding of the text of the convention. He points out that claims made

within the declaration that the convention would eventually allow germ-cell therapy, for example, are unfounded, as this is expressly ruled out in the convention.

"There were a lot of lies and misinterpretation of the convention which was not a lack of knowledge, but a political intention [to hinder genetics research]," he says. "I feel our history is being manipulated for this purpose".

Honnefelder argues that the convention, which has been considerably revised during the past two years, is extremely restrictive, particularly the clause allowing research on legally incapacitated persons. It specifies that there must be no alternative means for doing the research, whose aim must be restricted to a better understanding of the disease from which the patient suffers. The research must not harm the patient in any way, and must be stopped immediately if the patient objects.

Honnefelder points out that if no research at all were allowed, "there would be no possibility of clinical research in diseases such as Alzheimer's". But Wunder believes that improved medical knowledge should not be achieved at a cost to the human dignity of handicapped individuals.

German representatives in the Council of Europe's parliamentary assembly were the only source of significant objections when the bioethics convention was approved by the assembly, with some amendments, in September. It is now being discussed in committees of the German parliament. The subsequent parliamentary debate will lead to a proposal to the government on the mandate it should give its Council of Europe representatives in a final vote on the convention vote next month.

Alison Abbott

Russian academy opts again for Osipov

Moscow. Yuri Osipov has been re-elected president of the Russian Academy of Sciences (RAS) after a tense campaign in which he promised to support all branches of Russian science, despite the serious problems of under-funding.

Osipov had given a pessimistic report to the RAS presidium, saying that "the historical reserves of the academy's intellectual and material stability are fully exhausted". He pledged to make all branches of science "equal in poverty".

In contrast, his nearest rival in the election, Evgeny Velikhov, had promised to back "elite" science. He particularly wanted to support nuclear physics, and described subjects such as palaeontology as "trifles". Velikhov insisted that only viable areas of science should be saved from collapse. He promised to find new ways of financing research, but he failed to mention details.

The third candidate was Valentin Koptug, an RAS vice-president and chairman of the academy's Siberian branch presidium. Koptug is also a member of the central committee of the Russian Communist Party. He faced a heavy defeat and withdrew from the contest.

In the first stage of the election, the RAS presidium supported Osipov by 23 votes to 15 and recommended him to the general assembly as its candidate for president. Of more than 1,000 academicians who took part in the general assembly ballot last week, 777 voted for Osipov.

The general assembly opened with a speech from Viktor Chernomyrdin, the Russian prime minister, in which he said that "it was our tragic mistake at the beginning of Perestroika to consider science to be the same branch of the national economy as, say, industry". □

'Ambition and impatience' blamed for fraud

Washington. Fresh efforts to crack down on scientific misconduct could follow last week's revelations of extraordinary and systematic fraud by a graduate student in the laboratory of Francis Collins, director of the National Center for Human Genome Research (NCHGR) at the National Institutes of Health (NIH).

Collins was the senior author of five papers, each of which will be partially or wholly withdrawn in the wake of the fraud. His student is alleged to have fabricated data in studies of a genetic inversion that is believed to cause childhood leukaemia.

Speculating on why someone would do such a thing, Collins blames the impatient

ambition of the gifted. The brightest students sometimes lack patience with the tedious business of conducting experiments properly, he says. Perhaps tellingly, Collins says the student's father was "a scientist of some renown".

He adds that the student, who accompanied Collins when he moved from the University of Michigan to take charge of NCHGR on the NIH campus in Bethesda was exceptionally bright, "probably the most impressive you'd come across in ten years". Collins also denies that the work was inadequately supervised. "This wasn't somebody working away in a corner. It was a student I was very involved with."

The fraud was uncovered when an anonymous reviewer of a sixth paper, submitted to the journal *Oncogene*, noticed that a figure appeared to have been falsified.

Collins said that he had considered resigning his post at NCHGR after the fraud was uncovered in August, but had been persuaded by friends not to do so. "This is the worst nightmare a scientist has, that the truth could be undermined right under your nose," he says. "I knew that some people might question if I could continue to play an effective role as head of the centre, but I was encouraged by people whose judgement I value not to draw that conclusion."

Collins was due to address the annual meeting of the American Society of Human Genetics at San Francisco when news of the fraud broke in the *Chicago Tribune*. He prefaced his lecture with an explanation of the case that appeared to win the support of those present, whom he referred to as "my family".

But Kenneth Ryan, a professor of obstetrics at Harvard University and chair of a national commission that has called for tighter regulation of government-funded science, said the case raised questions about what supervision the student was receiving.

"My heart goes out" to Collins, Ryan added. "It could happen to anybody. But this is science's problem, and scientists should get their heads together and take it seriously." The US government is currently considering changes in the definition and handling of misconduct in response to the Ryan commission's recommendations.

Under current rules for investigating scientific misconduct, the case should have been kept secret until an investigation was completed by the University of Michigan and passed to the Office of Research Integrity, which would determine sanctions and make a public announcement.

But, in a move designed to minimize scientific fall-out from the case, Collins wrote to 100 colleagues on 1 October, outlining the sequence of events and listing the papers to be retracted. The letter did not identify the student. But his identity could be readily inferred from the papers, and he was named in the *New York Times* last week as Amitov Hajra.

A lawyer for Hajra told the newspaper that he would have no comment until the University of Michigan had completed its investigation. A spokeswoman for the university says that will take "several weeks".

Hajra had been studying the core binding factor beta (CBFB) gene, which is believed to combine with another gene, smooth muscle myosin heavy chain (SMMHC), to form a fusion protein which divides and multiplies to cause leukaemia. He and Collins were the only authors of a paper published in *Genomics* (26, 571; 1995) on ►

Scientists 'too quick' to back claims

Boston. David Baltimore, the Nobel laureate and professor of immunology at the Massachusetts Institute of Technology (MIT), last week criticized scientists who had been "much too quick" to support allegations of scientific misconduct against Theresa Imanishi-Kari, his co-author on a 1986 paper in the journal *Cell*.

Imanishi-Kari, a researcher at Tufts University in Medford, Massachusetts, was acquitted of the charges earlier this year (see *Nature* 381, 719; 1996). In his first public appearance to discuss the case, Baltimore complained about the behaviour of the "self-appointed fraud-busters" at the National Institutes of Health (NIH), the bullying tactics of Congressman John Dingell and his staff, and what he called the error-ridden reporting of a "monolithic press".

Baltimore was speaking at a colloquium sponsored jointly by MIT's Science, Technology and Society programme and Harvard University's history of science department. He made various suggestions about an appropriate response to allegations of scientific fraud.

Such charges, he said, should be addressed in an even-handed manner. "If one approaches a case with a preconception that fraud has occurred, many steps in the scientific process which appear fraudulent merely reflect the personal and creative aspects of science." It is important to return to the presumption that fraud in basic research is rare because of the "near certainty" of it being detected, he added. "We do not need fraud police."

He admitted that both incidents and allegations of fraud occur occasionally, but rarely, and that the US federal government needs a mechanism to respond. "But such an investigation should not involve people who are interested in

proving guilt or non-guilt, nor should an accuser be taken on as an adjunct to the [investigative] committee's activities," he said after the meeting.

Baltimore pointed out that universities have to be able to respond fast, with an inquiry board sufficiently separate from both the work and the parties involved to be able to operate independently. "You need to safeguard everybody, both the accused and the accuser, and an ombudsman can do that," he said.

MIT has adopted more rigorous protocols for dealing with academic misconduct since charges were first brought against Imanishi-Kari. The guidelines are designed to protect the privacy of both those who report apparent misconduct and the alleged offenders. They instruct supervisors to bring such situations promptly to the attention of the institute's senior officials.

"One area where MIT and Tufts failed [in handling the Imanishi-Kari case] was in having a clear, written record of the proceedings," Baltimore said. "That wasn't surprising since, at the time, no-one had any conception that this would blow up in the way that it did." He now advises students to maintain detailed accounts of experiments so that "five or ten years from now, they'll be able to reconstruct everything that they did".

Journalists, Baltimore said, need to approach such cases with an open mind. "Reporters must look behind the situation and conduct their own investigation, and not just be the prisoners of leaks." One of the panelists at the colloquium, Malcolm Gladwell, who covered the case for the *Washington Post*, admitted that the press was "manipulated" and "pulled along in the creation of a controversy and sustaining of a controversy".

Steve Nadis

► the structure of the CBFB gene, which is to be retracted in full.

Another 1995 paper in *Molecular and Cellular Biology* will also be fully retracted, while two more extensive studies published in the *Proceedings of the National Academy of Sciences* on the behaviour of CBFB-SMMHC *in vitro* will be partially retracted, along with a fifth paper from the journal *Genes, Chromosomes and Cancer*.

When the problem with the figure in the sixth paper was brought to Collins' attention by the editors of *Oncogene*, it took him "about half an hour" to verify that it had been falsified, Collins says. There followed "two weeks of digging back" through laboratory records "collecting evidence that there had been systematic, long term fabrication" of results. The student admitted the fabrication, orally and in writing, Collins says.

Asked why he had not noticed the problem with the figure himself, Collins says



Collins: checking false figure took little time.

he thinks that it is unusual for people to look at figures with a view to finding misrepresentation. "I may be doing more of that from now on," he says, adding that he is "intensely grateful" to the referee for noticing the problem.

As for the question of whether anyone can supervise a laboratory and run a large operation such as NCHGR at the same time, Collins says that he ran his lab there much the same way as he had done at the University of Michigan. Supervision of its work was "just too important to me" to neglect.

He said that it was "hard to know" how much time had been wasted by other scientists following the retracted work, but that "it wasn't a hot topic that everyone was jumping on".

But Ryan says that it is the loss of public trust, rather than the waste of research money, that scientists should be worrying about. He believes that fraud is much more widespread than the community admits.

Ryan wants a system of formal quality control, analogous to that used in industrial production, introduced into federally-funded laboratories. "I don't think enough is being done — and I've been beaten up pretty badly for saying so," says Ryan, referring to the community's hostile reaction to his commission's findings.

Even the severe critics of scientific conduct, however, congratulated Collins on his handling of this case once the fraud was detected. According to Walter Stewart, an outspoken critic of scientific misconduct who works at NIH, "on the facts as we know them now, the community owes him a great debt for doing the right thing in the most difficult of circumstances". **Colin Macilwain**

British ruling supports legal challenge to broad patents

London. Britain's House of Lords delivered its first legal judgement on genetic engineering patents last week, and gave a significant boost to the efforts of the European biotechnology industry to limit the breadth of protection that can be claimed for a single invention or discovery.

The move came as part of a decision by the Lords, which acts as Britain's highest legal authority, to uphold an appeal court decision invalidating a patent issued to Biogen Inc., based in Cambridge, Massachusetts, for a method of producing vaccines for hepatitis-B using genetically-engineered antigens.

The patent was based on research carried out by Kenneth Murray of the University of Edinburgh in the late 1970s. The immediate beneficiary of last week's decision is the British company Medeva, which plans to market its own hepatitis-B vaccine next year. Although this is based on a different genetic engineering technology, Medeva had been sued by Biogen for patent infringement.

But the decision has much broader implications, as it addresses what many in the biotechnology industry see as a major weakness in European patent legislation: the fact that, under the European Patent Convention, once a patent has been granted it cannot be challenged on the basis of the breadth of its claims.

Last year, the House of Commons select committee on science and technology, in its report on human genetics, recommended that the convention be "redrawn" to allow patents to be challenged on the grounds that their claims go too wide.

This proposal reflects a widespread view in the industry that, particularly in the early days of genetic engineering, several patents of questionable breadth were granted. One controversial example is the broad claim that has been granted to W. French Anderson and his colleagues in the United States for *ex vivo* techniques of gene therapy (see *Nature* 374, 393; 1995).

Attempts earlier this year by representatives of the biotechnology industry to persuade the European Patent Office, which is responsible for issuing Europe-wide patents under the convention, to change the rules on patent challenges were rebuffed.

But last week's ruling appears to move a substantial distance in this direction. Lord Hoffmann, the law Lord who wrote the ruling and is himself a former judge, appears to establish — at least under British law — that, even though the breadth of a patent claim is not explicitly listed as one of the aspects on which a patent can be challenged, questions of excessive breadth can nevertheless be used as the

basis for mounting such a challenge.

Hoffmann emphasized in his ruling that care was needed "not to stifle further research and healthy competition by allowing the first person who had found a way of achieving an obviously desirable goal to monopolize every other way of doing it". And he added: "The Wright brothers had shown that heavier than air flight was possible, but that had not entitled them to a monopoly of heavier-than-air flying machines."

Gerald Kamstra, a patent attorney with the company Simmons and Simmons in London, says: "This decision goes some way to achieving what the industry has been seeking, at least in the United Kingdom."

Similarly, Nicholas Scott Ram, a senior executive with British Biotech and an adviser to the Bioindustry Association, the industry lobby group, welcomes what he calls "the right decision". Scott Ram adds: "It puts a handle on the question of the scope of a claim, and helps to ensure that the reward represented by a patent is commensurate with the work put into it."

The House of Lords' judgement effectively overturns an initial ruling by a patent judge that Medeva's technique for producing hepatitis-B antigens, although based on very different technology (including full knowledge of the genomic sequence of the virus, which was unknown at the time of Holmes' work), did indeed infringe the Biogen patent.

Medeva managed to have this ruling overturned by the Court of Appeal in 1994. The appeal court ruled that the patent was invalid both because the techniques that Murray had used to obtain hepatitis-B antigens using recombinant DNA technology were "obvious" at the time, and because the claims in the patent application, seeking the rights to all recombinant DNA molecules coding for hepatitis-B antigens, were too broad.

Biogen appealed against this ruling to the House of Lords, which upheld its claim that the techniques used by Murray were in fact novel enough to justify the patent. But the Lords also judged that the patent application had gone too far, and that as a result the patent was invalid.

A spokeswoman for Medeva — which acquired the rights to its own drug, currently in phase three clinical trials, from Swiss and German scientists in 1992 — welcomed what she described as a "decisive victory" for the company. Officials of Biogen said that the company was "clearly disappointed" by the decision, but would continue to collect royalties on British sales of its own vaccine.

David Dickson

Brussels inquiry criticizes BSE secrecy

Paris. Serious flaws in the management of bovine spongiform encephalopathy (BSE) by the European Union (EU) are emerging from an inquiry into the 'mad cow' crisis by the European Parliament. Testimony given to the inquiry points to the failure of the European Commission (EC) to monitor adequately the enforcement of BSE control measures by member states, and to excessive secrecy in the way the commission makes decisions.

The inquiry is likely to recommend that the management of animal and public health within the EU's institutions should be brought under consumer protection rather than agriculture as at present, according to one source close to the inquiry committee. The source says the current set-up has been a recipe for conflicts of interest, where agricultural priorities have tended to prevail over those of public health.

The committee of inquiry has focused in particular on allegations that the commission suppressed information on BSE, and on evidence that UK exports of meat and bone meal increased substantially after the government banned its use in ruminant feed within the United Kingdom in 1988 (see *Nature* 381, 544; 1996).

The commission has come under fire following revelations in a memo written by one official, Gérard Castille, which records how an unnamed commission official had recommended "minimizing the BSE affair by practising disinformation" and "officially asking the United Kingdom to stop publishing its research".

The commission has argued that the memo reflected the view of an isolated civil servant, and not commission policy. One source close to the inquiry says it has found no firm evidence of a deliberate cover-up.

The commission has also provided a strong defence against allegations that it failed to prevent the threat posed by contaminated meat and bone meal. In July 1989, it proposed a ban on the export of meat and bone meal from the United Kingdom. But Britain refused, according to Lars Hoelgaard, head of the EC agriculture directorate's department of quality and health. He says that the commission then tried to introduce a European Community-wide ban on feeding ruminant protein to ruminants.

But this proposal was rejected by member states and by the commission's scientific veterinary committee. So the commission could only advise member states to introduce individual feed bans. Only France, Ireland, Denmark and the Netherlands did so. Other member states, including Germany, reacted only after the commission used its powers under the European single market to introduce a community-wide ban in 1994.

Hoelgaard says: "Here was a case where we didn't have the scientific evidence or the

powers to introduce a ban [in 1989], but where we saw feed was the cause and asked member states to impose their own bans."

The council of agriculture ministers of the member states comes under fire in the inquiry's draft conclusions for having pursued "minimalist policies" on BSE. Recent evidence of this comes from a meeting of ministers last week that rejected a proposal from the commission for stricter precautions against BSE in the light of a study showing that the new variant of Creutzfeldt-Jakob disease (CJD) was similar to BSE (see *Nature* 383, 685-690; 1996). The commission's proposed measures had been endorsed by its scientific veterinary committee.

Franz Fischler, EU agriculture commissioner, described the measures — which included a ban on sheep offals — as "absolutely necessary", arguing that "the precautionary principle must be an absolute priority". But several member states argued that the measures would penalize their economies even though they did not have BSE epidemics. The commission will re-submit its proposals to agriculture ministers at their next meeting later this month.

These events are a reminder that it is the council of ministers that ultimately pulls the strings of power in the EU. Ken Collins,

says Collins. He adds that if the commission had not adhered to scientific advice it could have been taken to the European Court of Justice or the World Trade Organization for blocking free trade. "In retrospect, the commission was too reluctant to take on the member states," he says.

One example of this is the failure of the commission to sufficiently monitor the implementation of BSE control measures in Britain. It began monitoring this in the first half of 1990 as part of routine inspections to ensure that abattoirs producing meat for export met EU standards. But all BSE inspections then ceased until 1994.

Guy Legras, director-general of the EC's agricultural directorate, DG6, has attributed this failure to understaffing of the commission's veterinary inspectorate, which had only a dozen individuals, compared with the estimated 200 required. "We did a poor job on inspections," admits Hoelgaard, who describes the sudden cessation of inspections as a "mystery".

The inspectors thought that they only had to inspect BSE control measures in Britain as a one-off exercise in the first half of 1990, says Hoelgaard, whereas he had understood it as a "standing instruction".

But a more sinister explanation emerges from reports commissioned recently by Hoelgaard from his inspectors. These allege that when the inspectors presented evidence in June 1990 of shortcomings in Britain's enforcement of BSE control measures — such as failure to remove nerve tissues adequately — Keith Meldrum, the United Kingdom's chief veterinary officer, "exploded", according to Hoelgaard. Meldrum told the inspectors that they "had no mandate to investigate BSE controls", he says. Meldrum is said to have claimed that "BSE is a political not a technical matter" and that "UK certificates are the best in the world".

Collins says: "You need commissioners that can stand up to pressure from the member states", and ensure that regulations on health are enforced. BSE has shown that the principle of subsidiarity is flawed when it comes to health issues, says one official from the parliament. Collins argues that, while subsidiarity allows for the devolution of responsibilities to member states, the commission has a duty to ensure that regulations are respected. This means having "a strong inspection system that can override states, and that means a strong agriculture commissioner — which we now have".

A wider question is the extent to which the commission and member states have allowed economics to take priority over public health. The commission's line is that it has followed science all the way, and that it has sometimes gone beyond the science. But this interpretation is questioned by some observers, who protest at what they ▶

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Holt Studios/Nigel Cattlin

Next in line? EC officials claim that a ban on sheep offals is 'absolutely necessary'.

chairman of the European Parliament's committee on environment, public health and consumer protection, says: "The more you look at the *realpolitik* of how the commission relates to the member states, you realize it has had to watch its step all the time."

Collins, who has often been an outspoken critic of the commission, says: "With BSE, the commission has been in the position of 'damned if it does and damned if it doesn't'." Had the commission taken tougher action against Britain earlier, the UK government would have gone to the media saying "look at these unelected bureaucrats" jeopardizing our beef industry,

► claim is excessive secrecy surrounding the commission's scientific committees, and question their legitimacy.

EU measures on BSE must be approved by either the standing veterinary committee — made up of the chief veterinary officers of member states — or the agriculture council. The commission submits proposals to them after taking advice from its scientific veterinary committee (SVC), made up of scientists selected by the commission from nominations by the member states. The SVC itself has a working party on BSE, made up of outside experts.

The fact that members of the SVC are appointed by member states undermines its independence, argue critics. "While the commission says it relies on science, one wonders how objective this science is within these committees," says Collins. "People on these are almost all appointees of national governments and in some sense represent their government's points of view."

Such concerns are heightened by the secrecy in which the committees operate. "The problem is to know what the hell is going on in these committees," says one official at the parliament. Allegations of excessive secrecy are confirmed by Hoelgaard, who himself says: "Speaking as Dane, I have been quite shocked and amazed by procedures in the commission."

The competence of some members of the SVC is also questioned by some scientists. One recalls a meeting of the SVC in July on

the risk of BSE in sheep. The SVC's working party on BSE had prepared "an interesting and rich working document", according to the observer, but it passed over the heads of most of those present.

The document was made available to the SVC only at the meeting, and committee members lacked time to consider it. The discussion, claims the observer, was "psychedelic" with "almost half" the members failing to grasp that it concerned the risk of BSE in sheep, and not scrapie.

The creation last summer of a multidisciplinary committee on BSE to advise the commission is an admission of the flaws in the scientific veterinary committee, according to observers. "The commission realized that it had to diminish the excessive power of DG6 [the agriculture directorate]," says one scientist involved.

Evidence of interference by DG6 comes from a stormy meeting of the industry directorate's scientific food committee on 8 March, almost two weeks before the UK government announced a possible link between BSE and CJD. One member of the committee says that Brian Marchant, a civil servant in DG6, disputed the meeting's

decision not to take a position on the unilateral exclusion by France and Germany of certain bovine materials from baby food, because it considered that the risk of BSE could not be excluded. Marchant "treated us as if we were incompetent and thought we were wrong to take a position that was different from the scientific veterinary committee", he says.

One outcome of the BSE crisis, admits one commission official, is that it has vindicated the Parliament's long-standing criticism of the system of committees. The BSE crisis has exposed that this system means that it "is hard to see who is responsible" for decisions.

A wide-ranging reorganization of the commission services is likely to result from a confidential audit of DG6's veterinary services commissioned by Legras from the commission's general inspectorate, which reports direct to Jacques Santer, the commission president.

One official from the parliament's environment committee says it is keen to have animal health and its consequences for public health moved out of DG6. It wants this activity to be combined with health — currently part of the directorate for employment and social affairs (DG5) — and shifted to the directorate for consumer protection (DG24). Only in this way can "the inherent conflict of interest" of handling health matters within the powerful agriculture directorate be avoided, he says. **Declan Butler**

IMAGE
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Meldrum: 'exploded'
over EC inspections.

Australian reef fisheries project swims for survival

Sydney. A decision by the Australian government to approve a project designed to evaluate the effects of line fishing and the rates of recovery of commercial and recreational species on the Great Barrier Reef (GBR) is being challenged in the federal parliament.

If the project proceeds, it will be the first controlled test of 'adaptive management' of coral reefs and the first in a marine World Heritage Area. Opposition to it focuses on the procedures for assessing the environmental impact of the survey, and on an alternative proposal for closing reefs as a precautionary measure.

Led by Bruce Mapstone, a biologist at the Cooperative Research Centre for Reef Research (CRC) in Townsville, Queensland, the programme is planned to last between five and ten years, and will be run in collaboration with the commercial and recreational fishing industries on 24 reefs selected from 3,000 within the 2,000-km-long GBR Marine Park.

In each of four clusters of six reefs, fishing will be allowed in two 'green reefs', currently closed to fishing. Two 'blue reefs' now open to fishing will be subjected to greater pressure for 12 months before being closed, and two reefs will be controls. After a year of measured harvesting, coral trout

(the basis of the industry) are expected to decline by 50 to 60 per cent, and the fished reefs will be closed for five years to measure their rates of recovery.

Fisheries around the world experiencing sudden collapses have done so without warning. The GBR experiment follows studies by Campbell Davies of the CRC, showing that coral trout do not migrate between different reefs. It is designed to measure how vulnerable or robust a fishery is under the pressure of fishing, and the effectiveness of reef closures as a means of replenishing stocks.

The experiment is supported by the Australian Marine Sciences Association, and has been approved by Robert Hill, the Minister for the Environment. But there has been vehement opposition from local conservation groups, as well as the Australian Democrats Party in the Senate. They claim that Hill by-passed a legal requirement to take note of advice from statutory authorities — which he denies.

Meg Lees, the Democrats' environment spokeswoman, is seeking to reverse Hill's change of zoning regulations for the GBR, attacking him for bowing to pressure from the commercial industry and asserting that his policies "would rip the heart out of some of the few protected regions of the GBR".

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All at sea? Critics point to conservation risks.

There have been only three other experiments in adaptive management, two in Canada and one in the Philippines. Carl Walters of the University of British Columbia, Canada, says that there is no direct experimental evidence to support adaptive management of reef fisheries. Most of the reefs in the GBR are now at risk of impacts from recreational and commercial fishing, he says. "We do not understand those impacts, and cannot model them correctly with available information." **Peter Pockley**

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AIDS researcher loses libel fight against French newspaper

Paris. Robert Gallo, the US AIDS researcher, has lost a battle against the newspaper *Le Monde* in the French Supreme Court of Appeal. The court threw out charges of libel and slander brought against the newspaper and its journalist Frank Nouchi in connection with articles written by Nouchi in 1991 on the dispute between Gallo and Luc Montagnier of the Institut Pasteur over claims on the discovery of the AIDS virus.

Nouchi based his articles on a draft report by the US National Institutes of Health's Office of Scientific Integrity that had been revealed by the *Chicago Tribune*. Nouchi's articles alleged that Gallo had censored his assistant Mikulas Popovic, and in so doing "contradicted the most elementary rules of scientific communication", and that he had used the virus discovered by Montagnier to develop the US AIDS test.

The court said that Nouchi had carried out a "serious and profound enquiry" into the Gallo/Montagnier dispute over the years, and had made every effort to corroborate the draft report's findings. The court ordered Gallo to pay legal costs incurred by *Le Monde*. □

'No evidence' for cable hazards

Washington. A panel of experts set up by the National Research Council has advised the US government that there is "no conclusive and consistent evidence" that exposure to electric and magnetic fields from overhead power lines causes cancer or other adverse health effects. The panel said the established correlation between the incidence of leukaemia and proximity to the lines was most likely to be due to other factors related to the vicinity of the power lines, such as poverty or high traffic density.

According to panel members, the study could help to dispel the widespread but unproven public perception that overhead power lines are a health hazard. But although some members said last week they themselves would comfortably live near the lines, others contended that until the cause of the correlation was established, research into a possible link should continue. □

First for Irish science policy

Dublin. The Irish government last week published its first white paper on science, technology and innovation. It lays down mechanisms for establishing a formal science policy, the absence of which had been blamed for the virtual disappearance of funding for basic research three years ago (see *Nature* 364, 662; 1993).

The white paper is based on a detailed report prepared by the Science, Technology and Innovation Advisory Council, made up of six academics, nine industrialists and three civil servants, which was established last year. Most of the report's 160 recommendations (see *Nature* 374, 396; 1995) have been adopted, including the idea of setting up government science planning committees. Although the white paper does not promise to raise the level of funding for research and development, some additional funds have already been injected into basic research this year. □

China aims at Moon missions

Hong Kong. China aims to send astronauts into space early next century and to devise cheap transport systems that could land people on the Moon, the National Space Administration said last week. Wang Liheng, vice-administrator of the space agency, said that China "expected to make a breakthrough" in crewed space flights in the next five years. He was speaking shortly before the opening of one of China's largest space fairs in Zhuhai.

The country is also developing light spacecraft for firing satellites into orbit and larger Long March rockets to carry payloads of up to 20 tonnes — double the current capacity — according to Li

Jianzhong, president of the China Academy of Launch Vehicle Technology. Cheng Fangyun of the Chinese Academy of Sciences said that China was close to developing a small spacecraft with low operating costs that could carry astronauts. "China has reliable technology for space tracking, telemetry and control to do this," he said. □

Neuroscientist gains top honour

Tokyo. Japan's leading neuroscientist, Masao Ito, capped a successful year last Sunday when he received the Order of Culture, Japan's highest award from the Emperor Akihito, given to recognize contributions to culture and learning. Ito, who is chairman of the Science Council of Japan, succeeded earlier this year in persuading the government to spend billions of extra dollars on brain research over the next 20 years (see *Nature* 382, 105; 1996).

The new nationwide initiative will be centred on the Institute of Physical and Chemical Research in the north-west suburbs of Tokyo where Ito heads the Frontier Research Programme and its brain research project. □

Russian suicide over finances

Moscow. Vladimir Nechai, director of the All-Russia Research Institute for Technical Physics, shot himself last week in apparent despair over its financial plight. The institute, outside the city of Chelyabinsk, had had its bank accounts seized by the local tax office earlier this year because of its failure to pay its bills for electricity and other local services.

Colleagues of Nechai say that, if this had not happened, the centre would have been able to continue to support itself, despite the fact that it has not received its government grant for several months. It has recently diversified away from research on nuclear weapons to such areas as artificial hearts and contact lenses. Staff say that the final straw for Nechai — who left no public explanation for his suicide — could have been a demand from Moscow that the institute should sack half its permanent staff. □

UK nanotech funds 'too small'

London. British research into 'nanotechnology' — the study of small-scale engineering structures — risks being left behind its competitors because of a lack of government funding. This is partly because it was not identified as a priority in the recent Technology Foresight exercise, says a report published this week by the Parliamentary Office of Science and Technology.

The report points out that by the end of the decade the global market for nanotechnology products may be worth as much as US\$80 billion. It says that new funding has dried up from both the National Initiative on Nanotechnology and a Nanotechnology Programme backed jointly by the Engineering and Physical Sciences Research Council and the Department of Trade and Industry. As a result, it says, "there is a danger that the momentum generated by these earlier programmes will be dissipated". The omission of nanotechnology from the Foresight exercise means that "scientists face an uphill struggle for funding". □

Japan eyes an end to tenure

Tokyo. Japan's University Council, an advisory body to the ministry of education, last week submitted a report to the ministry recommending the introduction of limited-term contracts for university faculty members. At present, all faculty members who are Japanese citizens automatically get lifetime employment.

The ministry plans to submit a bill in the next ordinary session of the Diet (Japanese parliament) in January to allow the introduction of contract employment. But it will be up to each university to decide whether to introduce the new practices. Strong resistance to the erosion of tenure among the academic community means that contracts are unlikely to be widely adopted unless financial incentives are offered to universities (see *Nature* 383, 654; 1996). □

Change comes slowly to South Africa

Reviving the fortunes of South African science after years of political isolation is proving an uphill task. The needs are enormous, while funding remains as tight as ever. Nevertheless many researchers remain optimistic.

HALF-WAY through its five-year term of office, South Africa's Government of National Unity is still far from meeting the high hopes of its scientific community that the ending of apartheid would lead to a blossoming of research activities.

Part of the reason has been financial. Not only has the economy remained weak, but — perhaps understandably — basic research has not been at the top of the new government's economic priorities. But there have also been political factors at work.

One reason for the initial high hopes, for example, was the creation by President Nelson Mandela of a Department for Arts, Culture, Science and Technology (DACST). But the ministerial portfolio was allocated to the Inkatha Freedom Party (IFP), the not-too-cosy bedfellow of the African National Congress. Indeed, it now appears that the creation of a specific portfolio for science and technology may have paradoxically undermined their development.

The first minister, Ben Ngubane, a physician, proved very effective at charming foreign governments. But he was less successful in persuading his own government to reverse trends in research spending. His successor, Lionel Mtshali, has been in office for only two months, and, unsurprisingly, has so far made little impact.



Mandela: created new science ministry.

The political reality is that Inkatha remains marginalized — and thus relatively powerless — in the cabinet, and that despite the best efforts of both incumbents, research has not escaped unscathed.

Yet despite the difficulties it faces, South African science is far from withering, and is certainly benefiting from wider international recognition and acceptance. "Although science wasn't nearly as badly hit by the academic boycott as the humanities, the real worth of many South African scientists is now being recognized in many international fora," says Cliff Moran, dean of the science faculty at the University of Cape Town.

Briefing was written by **Michael Cherry**, *Nature's* South Africa correspondent. Current exchange rate: US\$1=R4.68

But Moran cautions that "the declining rand has had a more important effect than the advent of democracy on the state of science in the country". In fact, the rand has dropped steadily against most international currencies, including both the dollar and the pound sterling. This collapse in value has, in particular, had a crippling effect on the cost of scientific equipment, most of which is imported, and on the purchase of international journals.

There are plenty of optimists. "You shouldn't underestimate the power of fairy tales," says Alec Erwin, minister of trade and industry. But, despite South Africa's remarkable achievement of political reconciliation, the country's economic recovery has been agonisingly slow, and its prolonged recession, from which it is yet to emerge, has hit research hard.

Between 1985 and 1991, spending on research and development stood at about one per cent of the gross domestic product. But figures released earlier this year show that in 1993/94, the last year of the National Party government, this figure had dropped to 0.75 per cent.

The drop in funding has been matched by a decline in South Africa's share of the international production of research papers, as reflected in publications listed by the Institute for Scientific Information (ISI) in Philadelphia. This rose constantly during the 1980s, peaking in 1987, with particular strengths in fields such as botany, zoology and clinical medicine (see page 13).

But according to Anastassios Pouris, an independent science policy consultant, this share of the world's scientific output has been in decline ever since, and countries like China, Taiwan, Brazil and Norway, which were either below or on a par with South Africa 15 years ago, have now passed it.

Furthermore, while the number of papers listed by ISI has been constant since 1987 — at approximately 3,300 a year — more than 60 per cent of these publications are now produced by researchers at only five universities: Cape Town, Witwatersrand (in Johannesburg), Pretoria, Natal and Stellenbosch (near Cape Town). By comparison, says Pouris, the seven research councils, which employ the majority of the country's researchers, produce fewer than 200 ISI publications a year between them.

Many South African scientists acknowledge that there are some areas of science in which the country cannot pretend to compete with western countries. "We have to identify market niches in science and go

for them," says Moran at Cape Town. "I am confident that we can do this successfully with the resources at our disposal," he adds.

The need to identify scientific priorities for South Africa has been accepted by the

SAAG

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Aiming high: the South African Astronomical Observatory's 1.9-m telescope in Sutherland.

new department, which has embarked — at great expense — on two ambitious projects: an audit of all the country's research activities, and a foresight exercise, which is intended to "identify those areas of science and technology that are likely to yield the greatest economic and social benefits for South Africa in the longer term".

Many hopes have been attached to a new white paper on science and technology, published in September (see page 12). But the changes implied by this document have yet to be effected. Similarly, a National Commission on Higher Education has made proposals for significant reforms in this sector. But their implementation is likely to be thwarted by political and other difficulties.

But despite the challenges still faced and the slow progress experienced so far, many South African scientists remain optimistic that a renaissance is still just around the corner. □

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Hopes rise for redistribution of funds

THE future of research in South Africa is likely to depend heavily on the impact of a government white paper on science and technology, approved by the cabinet in September, which will bring major organizational changes and signal greater reliance on private-sector research.

The initiative has been welcomed by the scientific establishment as an attempt to secure a more effective distribution of the science vote, concentrating funds on the most productive areas of research. George Ellis, president of the Royal Society of South Africa, says that he is "very upbeat" about the white paper, which he describes as a "terrific statement".

But others doubt whether, given the general and continuing decline in funding, the reforms proposed in the paper will be sufficient to ensure the future health of South African science.

Three major policy changes are being proposed. The first is that a National Research Foundation (NRF) should be given responsibility for all research carried out by universities and other tertiary educational institutions.

The organizational would be created by merging the Foundation for Research Development (FRD), South Africa's main funding agency for science and technology, with the much smaller grant-giving responsibilities of the Human Sciences Research Council (HSRC).

The second change is the creation of a National Innovation Fund (NIF), operating under the aegis of a National Advisory Council on Innovation. This is intended to stimulate innovative research, and respond rapidly to changing research and technological priorities.

The third change is that the activities of the country's seven science councils will be regularly reviewed by both scientists and end-users. This will effectively replace the current system of relative autonomy, under which the councils set and follow their own agendas without having to account publicly for how they spend their money.

At present, 57 per cent of the science vote (see table, below left) is spent — much of it relatively unproductively — by the Agricultural Research Council and the Council for Scientific and Industrial Research. Under the white paper's reforms, the budget will now be redistributed among the various research councils, the NRF and the NIF.

In addition, both the research councils and the NRF will be able to apply to the NIF for additional funds for specific programmes. Overall responsibility for deciding how the money is divided up will lie with the Ministerial Committee on Science and Technology (MCST), a cabinet committee on which all ministers whose portfolios have a research component will be represented.

How effective will the reforms be? Many scientists warn that organizational changes will not be sufficient to stimulate South African science. Khotso Mokhele, president of both the FRD and the Academy of Sciences of South Africa, warns that agency funding has fallen to critically low levels.

Within the FRD core programme, for example, the average value of grants to university researchers fell from R48,000 to R20,000 (at 1989 values) between 1989 and 1995. The real value of grants has decreased even more, as a result of the declining exchange rate over this period.

The government plans that funding redistribution will be made for the first time in 1998, by which time all new institutions involved in allocating funds to researchers and their institutions should have been set up. Rob Adam, deputy director-general in the Department for Arts, Culture, Science and Technology, says that comprehensive reviews of the activities of each of the councils will be carried out next year, and assessed before reallocations are made.

Mokhele is enthusiastic about the proposed single funding agency, the NRF, "provided that peer review is retained as an essential component of the system". He concedes that the focus of such a review need not be a rating of the scientist submitting the proposal — as currently practised by the FRD — but that the project proposal itself could become more important. "But all this will have to be decided by the new foundation," he says.

The foundation will have four divisions. These will be responsible for natural sciences and engineering; social sciences and humanities; health sciences (encompassing the medical and health-support work of the FRD and HSRC, but not the Medical Research Council, MRC); and agricultural and environmental sciences (including the agricultural projects of the FRD, but essentially a new component of funding).

Interestingly, the Department of Health

appears to have won a battle to prevent the grant-awarding responsibilities of the MRC being taken over by the NRF. "Our contention is that in health research it is optimal to integrate in-house functions with those performed by the tertiary education sector," says Tony Mbewu, the MRC's group executive for research.

Ironically, although the white paper assumes that the focus of investment in R&D needs to shift away from government-funded activities to those in which the private sector has an interest, it does

not suggest incentives to encourage this. "The engagement of the corporate sector has yet to be fully secured," says Nick Segal, president of the Chamber of Mines.

The white paper rejects the suggestion of tax breaks for private-sector R&D. The govern-

ment's explanation is that its revenue collection services lack the capacity to administer such a system. Equally plausible, say some, is that an Inkatha Freedom Party minister does not carry enough weight in cabinet to prevail over the minister of finance. But Lionel Mtshali, the minister responsible for science, says he "will put up a fight for tax concessions, both for museums and private-sector R&D in science and technology".

Both military research funded from the defence budget — currently costing R500 million — and the activities of the Atomic Energy Corporation (AEC), which this year had a subsidy of R345 million from the Department of Mineral and Energy Affairs, are likely to come under scrutiny.

One specific recommendation of the white paper is that the AEC's SAFARI-1 nuclear reactor should be declared a national facility. The proposal, which originated from the corporation itself, will formalize the arrangement whereby university scientists have free access to the reactor.

The political symbolism of declaring SAFARI-1 — which is being loaded with highly-enriched uranium from South Africa's now-dismantled nuclear weapons — a national facility is lost on no-one. But the wisdom of the decision has been questioned by some of the country's physicists, including Fritz Hahne, dean of the science faculty at the University of Stellenbosch. If it becomes necessary to choose between SAFARI-1 and the controversial National Accelerator Centre (see page 13), he says, "the latter has far more potential". □



Mtshali: supporting calls for tax breaks.

Allocation of the 1996/7 Science Vote (US\$1 = 4.7 rand)

Council	Millions of rands
Agricultural Research Council (ARC)	296
Council for Scientific and Industrial Research (CSIR)	267
Human Sciences Research Council (HSRC)	88
Foundation for Research Development (FRD)	86
Council for Minerals Technology (Mintek)	73
Council for Geosciences	60
Medical Research Council (MRC)	58
National Accelerator Centre (NAC)	38
South African Astronomical Observatory (SAAO)	9
Hartebeespoort Radio-Astronomical Observatory (HRAO)	5
Total	980

Research flourishes, even in harsh climate

DESPITE its recent political isolation and limited funding for research, South Africa has managed to maintain a world-class scientific tradition in several disciplines. Bibliometric and citation-based surveys tend to confirm the view in the scientific community that the country is particularly strong in four fields: organismic biology, Earth sciences, astronomy and clinical medicine.

The topics are not surprising, given the country's situation and its natural environment. South Africa has remarkable biodiversity, and an interesting geological history, which have provided the focus of botanical, zoological and geological studies. And its excellent viewing conditions have made it the focus of southern hemisphere astronomy since Herschel first visited the Cape in the 1830s.

In addition, a national shortage of water has prompted research on water resources, while the importance of the mining industry ensures that metallurgy is well supported. Finally, the existence of world-class medical facilities has enabled South African clinicians to make greater advances than elsewhere in the continent.

But there remain significant disparities between the distribution of South Africa's research budget (see table, opposite page) and the disciplines in which the country has demonstrable scientific strength. Apart from the activities of the Atomic Energy Corporation, for example, the country's most important civilian capital project has been a 200-MeV cyclotron, opened 11 years ago at the National Accelerator Centre at Faure, 30 km east of Cape Town.

This is not a scientific field in which South Africa enjoys a particularly high international reputation. Yet recent cutbacks experienced by most research and tertiary institutions appear not to have applied to the accelerator centre. Its staff has increased by 10 per cent over the past two years, and its budget has risen by R3 million to R38 million.

The centre's new director, John Sharpey-Shaffer, who took up office a year ago, concedes that it is a bigger institution than one would expect to find in South Africa. "There are people who don't like it, and they have a good case," he says. The centre is currently being reviewed by a panel of experts including three foreign and two local members. Sharpey-Shaffer thinks that its future lies not as a privatized medical facility, but in heavy-ion research, and has spent R4.5 million so far in developing gamma-ray detectors for this purpose.

Astronomy has a better case for being treated as a legitimate 'big science'. The South African Astronomical Observatory (SAAO) has recently revised its plans for a new Southern African Large Telescope to replace the existing 1.9-metre telescope at

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

The King Protea is part of South Africa's rich biodiversity, a key factor of its science.

its observatory at Sutherland, about 400 km north-east of Cape Town. The original proposal was for a 4-metre class telescope using modern thin-mirror technology with active and adaptive optics (see *Nature* 372, 394; 1994).

Last month SAAO announced that it is now seeking an 11-metre spectroscopic survey telescope, similar to the Hobby-Eberly Telescope (HET) nearing completion at the MacDonal Observatory in Texas. Case Rijdsdijk, the SAAO's education officer, says that this could be produced at

an estimated cost of R90 million, two-thirds of the price of the 4-metre telescope originally proposed, as the hexagonal mirror comprises an array of 91 lightweight mirrors, which are relatively inexpensive to produce.

The design provides 70 per cent sky coverage, while keeping constant gravity's pull on the mirrors, so avoiding distortions that can compromise image quality. Moreover, it would not be overshadowed by other larger telescopes in the southern hemisphere, chiefly the new European Southern Observatory telescope under construction at Paranal in Chile.

The new instrument would provide a complementary facility for the HET in the southern hemisphere. According to Rijdsdijk, it would enable South Africa to retain its important position in world astronomical research.

The SAAO plans to construct the telescope at its observatory at Sutherland. It abandoned the idea of being involved in the development of the Gamsberg site, in Namibia, after the government indicated that it would be unwilling to invest in a site outside South Africa's borders.

Whether the government will be prepared to look more favourably at the new plan remains to be seen. But Lionel Mtshali, the new minister for arts, culture, science and technology, has assured his support for the project, which he regards as "an exciting development". The SAAO is asking the government for R10 million a year for the next five years, and hopes to raise the balance from foreign funding sources. □

All change for university funding?

SOUTH African universities appear to be entering a new period of uncertainty, following the publication last month of the final report of the National Commission for Higher Education, which proposes far-reaching changes in the financing of tertiary education and is likely to become the basis of government policy.

The report suggests how future growth in student numbers might be kept in line with the country's manpower requirements. Many agree that this will help to make up for the current poor correlation between the universities' graduate output and national needs. But critics point out that the report does not even promise to maintain existing levels of research funding for universities.

The commission has been chaired by Jairam Reddy, a former rector of the University of Durban-Westville. It proposes that in future the state should finance the research and teaching components of a university's subsidy separately, so that outputs from both can be accounted for independently. But it makes no specific pro-



Bengu: 'universities must be accountable'.

posals on criteria for research funding, apart from recommending that funds allocated by the government for research should not be diverted to other purposes.

Sibusiso Bengu, the minister of education — and formerly vice-chancellor of the University of Fort Hare — argues that his department is "committed to upgrading the research capacity of universities", and promises that incentives aimed at doing this will be incorporated in the new funding formula.

The government is currently preparing a response to the commission's report. Bengu says that a white paper will be published within the next few months, and that legislation will be put before parliament by next May. If it is approved, universities will be ►

► funded according to the new formula for the first time in 1998.

Many universities are already facing serious difficulties. Michael Smout, pro vice-chancellor of Rhodes University in Grahamstown, north-east of Port Elizabeth, points out that the (as yet undetermined) allocation of university subsidies for 1997, the last to be made under the old system, will be critical for the future of research. "If funding levels are cut next year, we will be unable to replace obsolete equipment and maintain library subscriptions to journals," he says.

The commission recommends that teaching funds should be allocated on the basis of fixed subsidies per degree place in each discipline, varying according to the costs of training a student in that discipline. The state would set the national total of subsidized places in each discipline, and

individual institutions would negotiate a share of these places for a three-year period.

This contrasts sharply with the present funding system, in operation since 1982, under which funding is based on a formula incorporating student enrolment and success rates, but with different weighting for arts and science-based courses and undergraduate and postgraduate students, as well as research output.

The lack of limits on the number of students in different disciplines has led to undirected growth over the past 15 years, concentrated in the arts and social sciences. This has been exacerbated by the fact that universities have been free to spend their subsidies as they wish. Bengu says his aim is not to curtail university autonomy, but simply to make universities accountable to government for different categories of funding.

The commission proposes that universities and technikons should be administered under one system, and financed in the same manner. But it leaves open the question of whether some differentiation between the two should remain.

Universities are being cautious about the planned reforms. Hugh Amoore, for example, registrar of the University of Cape Town (UCT), says that the government needs to provide more details about the new funding formula before it can be adequately assessed. He also warns that the proposed system will require a sophisticated infrastructure to implement it properly.

Ian Scott, director of academic support at UCT, adds: "Whether the government will have the political will to implement these recommendations in a sensible manner is another matter." □

Education reforms struggle against apartheid's legacy

NO-ONE envied Sibusiso Bengu when he was appointed minister of education in May 1994. The mild-mannered vice-chancellor of the University of Fort Hare, President Nelson Mandela's *alma mater*, took on responsibility for redressing what was widely perceived as apartheid's most invidious legacy — its education system.

Two-and-a-half years after his appointment, Bengu's progress has indeed been slow. For example, no curriculum reforms have been introduced in the syllabuses for mathematics, biology and physical science — even though this is widely recognized as essential if the country is to improve its scientific performance. This has been a source of frustration to scientists, many of whom worked hard to formulate proposals.

Admittedly, Bengu's task is enormous and complex. Almost 30 per cent of South Africa's population of 40 million are school pupils, and 18.5 per cent of the national budget is spent on them. His department, which also includes responsibility for higher education, is the biggest spender in the government.

One of Bengu's problems is that, unlike some of his cabinet colleagues, he has not been able to surround himself with competent officials. Those attending the launch of the Academy of Sciences of South Africa earlier this year were dismayed by the performance of a top civil servant in the department, who gave a talk presenting a vision of a new education system which some described as almost completely incoherent.

Bengu and the rest of the government are now pinning their hopes for educational reform on a bill that he has presented to parliament, and which is likely to be passed before the current session ends later this month.

The situation concerning education in mathematics and science is not encoura-

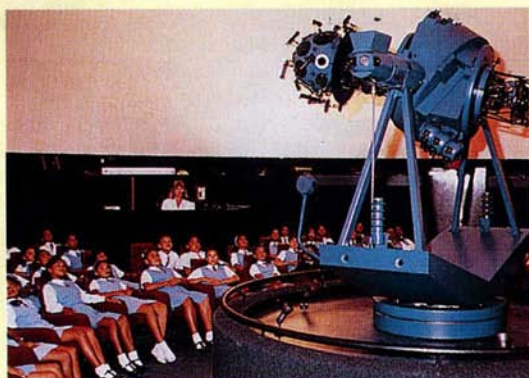
ging. A major problem is that only 33 per cent of African pupils choose mathematics as one of their six required subjects in their final (matriculation) year, in contrast to 73 per cent of whites. The figure for physical science is even worse, with only 12 per cent of Africans taking the subject, compared with 50 per cent of white pupils.

Furthermore, pass rates for African students in maths are less than a third of those for whites, coloureds and Indians, while pass rates in physical science and biology are around half those for the other population groups. The reason for this discrepancy is that, as mathematics is a prerequisite for taking physical science, those studying the latter are already a select group, and a higher percentage pass.

In contrast, biology is studied by a greater proportion of African and coloured pupils (more than 80 per cent) than whites (55 per cent). But the syllabus is dominated by rote-learning, and does not include molecular biology. Evolution is not studied either, reflecting the influence of creationists on the education policy-makers of the former regime.

Enrolments and pass rates are affected by several factors, the most important being the availability of qualified teachers. A third of African teachers in maths and biology are either unqualified or under-qualified, as are a quarter of those in physical science. Most significantly, half of the African teachers in general science fall into this category.

General science combines biology and physical science, and is offered for the first two years of secondary schooling. But poor teaching in black schools at this level



Future projections: curriculum reforms are vital.

clearly mitigates against the choice of physical science as a matriculation subject, while the biology syllabus, dominated by rote-learning, is often found to be more manageable by pupils.

Attempts by central government to eliminate wide disparities in pupil-teacher ratios between the provinces means that the Western Cape government is being forced to lay off 6,000 teachers this year to bring its ratio in line with the national average of 35:1 in secondary schools, and 40:1 in primary schools.

Generous redundancy packages have been offered. But, ironically, one effect is that many science and maths teachers, who are in demand in the private sector, are leaving education altogether, as a condition of the packages is that they do not re-enter government service, at national or provincial level.

Bengu says that he "doubts" that this is taking place, as the government has the right to refuse any individual application for a severance package. But he concedes that the process is being implemented by the provincial education departments, and admits that he is not intervening in the process in any way. □

Restructuring plans could revitalize museums' role

PARLIAMENT'S standing committee on arts, culture, science and technology recently visited the South African Astronomical Observatory at Sutherland. Having explained the importance of its work, Bob Stobie, its director, was upset to find that the main interest of the committee members was to engage him in a debate about astrology.

The anecdote indicates that many South African politicians appear as ignorant of science as their constituents. One potential remedy for the problem of scientific illiteracy lies in museums. Over the past decade, most of the country's natural history museums have developed outreach programmes, both to target adult education and to supplement school curricula.

Yet a white paper on arts and culture, approved by the cabinet in September, fails to address either the future of South Africa's natural history museums, or the foundation of a national museum on science and technology, which many feel might at least start addressing public ignorance.

But the paper does acknowledge that the national status of the 18 museums currently funded by subsidies from the Department of Arts, Culture, Science and Technology (DACST) — with a total value of only R49 million — needs to be reviewed. Many are not really national institutions — their status was elevated by the previous government for political reasons. After two-and-a-half years of government procrastination on museums, Themba Wakashe, chief director in the DACST responsible for museums, concedes that "a decision has to be made, as the museum world is demoralized".

Five of these institutions have major natural history collections, as do at least five provincial museums and one municipal museum — the Durban Museum of Natural

Science — which is the country's most popular museum. In addition, large collections of insects, nematodes, fungi and arachnids are held by the Agricultural Research Council.

Apart from the fragmentation of collections, one of the main problems facing the museums is that, with a few exceptions, their displays are outdated and lack information content. A rationalization survey conducted for the department by consultants Deloitte and Touche found them to be overmanaged in terms of posts. But perhaps even more critical is that most are in desperate need of new management blood. Restructuring might at least allow for new management to be brought in, with a brief to focus on the development of display programmes.

The report favours the model proposed last year by the Arts and Culture Task Group (ACTAG) of having two national museums, including natural history, in each of the country's twin capitals of Cape Town and Pretoria. But it has attracted criticism from biologists, who fear that this would result in downgrading the status of biological collections of national importance lying outside these two centres (see *Nature* 377, 5; 1995).

Non-national museums have been included in a review of all the country's museums, which has recently been completed, according to Wakashe. This task was undertaken by a four-member panel which included Wakashe himself.

Their recommendations were considered last week by the council of (provincial) ministers of arts and culture. They are now discussing the recommendations with the provincial governments, and there are strong indications that the provinces are going to be reluctant to relinquish their important collections to the national museums.

An additional decision has to be made on the foundation of a national museum of science and technology, suggested by ACTAG. There is already debate over its possible location. One suggestion is that it could be accommodated in some of the empty buildings that housed the now-defunct facilities at Pelindaba where the Atomic Energy Corporation enriched uranium for nuclear devices and "commercial purposes".

The corporation's chief executive, Waldo Stumpf, is amenable to the idea, but questions whether the location, 25 km west of Pretoria, would be optimal for attracting a large number of visitors. □

International links help to overcome years of isolation

THERE are several bright spots in the gloom that currently envelops much of South African science. One is that, as a result of the decision to abandon apartheid, the country is now emerging from a prolonged period of international isolation, both scientific and otherwise.

Foreign scientists are flocking to the country, both as visitors and as researchers. Some of the more prominent individuals in the first category have included both the immediate past and present presidents of Britain's Royal Society, Sir Michael Atiyah and Sir Aaron Klug, and Walter Massey, former director of the US National Science Foundation (NSF), who suggested to a South African audience last month that science could serve as a "cultural glue" to unite its multicultural society.

A number of bilateral co-operative agreements have been signed since the ending of apartheid with countries such as the United Kingdom, France, the United States and Germany. Many foreign sources of research and travel funds are becoming available to South African scientists for the first time, including European Union and Commonwealth Science Council funding.

Some Western research funding bodies have deliberately chosen to allocate funds to promote South African science, partly in the hope that this will have a broader impact on the region. Britain's Wellcome Trust, for example, has targeted South Africa because it "sees the country as providing it with a collaborative base for development in the developing African continent", according to Michael Wilkinson, its international scientific programme manager.

Wellcome has introduced a range of grant and fellowship schemes for collaborative exchanges between South Africa and the United Kingdom. The trust is also currently considering applications from four institutions in southern Africa to host a new Wellcome Centre for Population Studies. Other support from Britain has come in the form of a promise of five years' funding from the Royal Society to help build up research capacity in historically disadvantaged institutions.

Meanwhile, several senior administrators from the NSF have recently visited South Africa to discuss closer collaboration with US scientists. No specific funds are being set aside for this, but NSF officials say that proposals from US researchers for collaborative research projects are being actively encouraged. □

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No maternal transmission?

SIR — Recent reports in the media of the outcome of a long-term study of the incidence of bovine spongiform encephalopathy (BSE) in the offspring of cows affected with BSE have widely assumed that these data show evidence of maternal transmission of BSE. That maternal transmission occurs in BSE has been incorporated in a recent modelling of the epidemic of BSE¹, and data from this study and the supposition that maternal transmission occurs have been taken up by politicians in Britain and Europe in arguing about the best way to restore confidence in beef throughout Europe.

The use of these data is premature, as the full details of the maternal transmission experiment are not yet published. So far, we know from the Spongiform Encephalopathy Advisory Committee that, of 276 calves born to dams that subsequently died of BSE, 42 have themselves developed BSE, while, of 276 calves born to dams that did not get BSE, only 13 have developed BSE.

How might this difference have arisen? Three possibilities come to mind: (1) selection bias in the way these calves were acquired, (2) genetic susceptibility to infection with the agent of BSE and (3) transmission of the agent from the incubating (but not overtly sick) dam to the offspring. The numerical data, in the gross terms described, cannot differentiate between genetic susceptibility and maternal transmission. The calves involved were born around 1988, when exposure to contaminated feedstuff was high. The dams of these animals would also have been exposed to the feed-borne contamination which began in 1982. If there was a difference in genetic susceptibility to feed-borne infection between the affected and unaffected dams, this would also be apparent in the offspring, leading to a higher rate in the offspring of affected compared to unaffected dams. To show convincingly that maternal transmission was occurring, these offspring would have to have been unexposed to agent from feedstuff.

So why are the data interpreted solely as evidence for maternal transmission? The answer probably lies in the widespread belief in maternal transmission in scrapie which persists despite new data that indicate that "there is no strong evidence for simple maternal or paternal transmission of disease [scrapie] other than inheritance of *PrP* genotype"². Our reanalysis of the original data used to support maternal transmission shows no deviation from genetic inheritance of scrapie, and no evidence for maternal transmission in any other form of spongiform encephalopathy³. Furthermore, there is no established mechanism by which maternal transmission would occur. Data on infectivity in blood and placenta in

sheep^{4,5} are inadequate, and experiments have failed to detect infectivity in milk or any tissue outside the central nervous system in field cases of BSE⁶. Genetic involvement in prion disease is well established. In addition to point mutations in the *PrP* gene which determine disease, homozygosity at codon 129 of the *PrP* gene increases susceptibility to iatrogenic and sporadic Creutzfeldt-Jakob disease^{7,8}, and transgenic experiments have shown that homology between the prion protein molecules produced by each allele is important in pathogenesis⁹.

Although some analysis of the *PrP* gene in BSE-affected cattle has been undertaken¹⁰, the gene has not been studied extensively. Genetic influences outside the *PrP* gene may also be important in determining which of a very large number of exposed animals succumb to BSE. Epidemiological analysis of BSE suggests that genetic influences may be occurring, but this cannot be definitively proved¹¹. In these circumstances, it would be foolish to assume that maternal transmission occurs in BSE, especially when decisions of great economic and political importance are at stake.

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The problem with MAFF

SIR — It is encouraging to see your leading article¹ asking for a change in policy by the Ministry of Agriculture, Fisheries and Food (MAFF) over data on bovine spongiform encephalopathy (BSE).

Although it probably represents only a minor part of total research, MAFF has a history of suppressing scientific information it does not like, either by banning

publication by its research staff or suggesting to those outside that publication of certain MAFF-sponsored research might jeopardize further funding. This is probably a cultural problem within MAFF arising from the view that the role of scientific civil servants is to support politicians. As a result, science must be made to fit policy (which may be based on dogma), rather than policy being based on scientific understanding.

Dogma overriding scientific understanding is well illustrated by MAFF's attitude to the sheep industry. The United Kingdom has the largest and most successful sheep industry within the European Union (EU). Approximate values² indicate that there were 43,901,000 sheep and lambs in June 1993, which provided a total income, including that to sheep-associated industries, of about £3.5 billion a year, and gave employment to about 250,000 people. The EU guaranteed-support payment in 1993 was £422 million, but MAFF spends almost nothing on the most important lowland disease of sheep, infection with nematodes.

Because nematodes resistant to all three groups of anthelmintics have been found in the United Kingdom³, sooner or later the sheep industry will be forced to contract when total anthelmintic failure occurs on sheep farms. Not only is MAFF not interested, but the EU does not fund research in this area because "worms do not matter". MAFF will not pay directly, and appears opposed on political grounds to levies. Levies are the only other way of collecting money for essential research and farmer education programmes that are urgently required.

The only people who can change attitudes in MAFF are Members of Parliament. Scientists and farmers alike should write to their MPs and demand freedom of information on quality research sponsored by MAFF. In addition, because of changing diseases, MPs must insist on a national agricultural programme that is based on maintaining wealth generation (and food supplies) through increased research on disease control in plants and animals.

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Letters submitted for Correspondence can be e-mailed to **corres@nature.com**, but please send only items intended for the Correspondence section to this address.

Nicotine, tobacco and addiction

SIR — For many people, the concept of addiction involves taking of drugs¹; most official definitions of addiction include drug ingestion. Despite such definitions, there are many potentially addictive behaviours that do not involve drug ingestion, for example gambling, overeating, sex, exercise, computer-game playing, the Internet, pair bonding and work^{2,3}. Such diversity has led to all-encompassing definitions of what constitutes addictive behaviour. Trying to define 'addiction' is rather like defining a 'mountain' or 'tree' in that there is no single set of criteria that can ever be necessary or sufficient to define all instances. In essence, the whole is easier to recognize than the parts. Here I suggest six components that in my view need to be fulfilled if a behaviour is to be defined as 'addictive'.

Salience: when the particular activity becomes the most important activity in people's lives and dominates their thinking (preoccupations and cognitive distortions), feelings (cravings) and behaviour (deterioration of socialized behaviour). For instance, even if they are not actually engaged in the behaviour, they will be thinking about the next time they will be.

Mood modification: subjective experiences that people report as a consequence of engaging in the particular activity and can

be seen as a coping strategy (they experience an arousing 'buzz' or a 'high' or a paradoxically tranquilizing feel of 'escape' or 'numbing').

Tolerance: a process whereby increasing amounts of the particular activity are required to achieve the former effects. For instance, a gambler may have gradually to increase the size of the bet to experience a euphoric effect that was initially obtained by a much smaller bet.

Withdrawal symptoms: unpleasant feeling states and/or physical effects that occur when the particular activity is discontinued or suddenly reduced, for example 'the shakes', moodiness or irritability.

Conflict: conflicts between addicts and those around them (interpersonal conflict) or from within the individual (intrapsychic conflict) that are concerned with the particular activity.

Relapse: the tendency for repeated reversions to earlier patterns of the particular activity to recur and for even the most extreme patterns typical of the height of the addiction to be quickly restored after many years of abstinence or control.

I believe that explanations for addiction must come from a biopsychosocial approach, in that 'addiction' arises from a combination of biological predisposition,

social environment and psychological constitution. To many, this goes without saying, but others present oversimplistic and parsimonious explanations. Behavioral addictions *do* exist, and should be treated no differently from the better-known chemically based addictions.

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SIR — Although there is little doubt that cigarette smoking is hazardous to health and that nicotine plays a major role in reinforcing this behaviour¹, we believe that certain effects of nicotine itself need to be dissociated from tobacco and other drugs of abuse.

Despite recent findings² demonstrating common neuropharmacological and neuro-anatomical similarities between the effects of nicotine and other drugs of abuse, there are some important differences. For example, nicotine is typically devoid of the profound euphoric and perceptual effects offered by many drugs of abuse, and there is little evidence that the habitual consumption of nicotine (via tobacco) causes depression or psychosis³.



So far, systems in the ÄKTA design family include:

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- the new ÄKTA Purifier, for purification of peptides, oligonucleotides and other biomolecules

Unfortunately, the title of the *Nature* News and Views article⁴ about the new findings read "Smoking... harmful to the brain", when there was little if any evidence presented to suggest that nicotine itself is harmful to the brain. Although tobacco contains nicotine, it also contains many harmful chemicals in the tar and smoke. It is likely that the increase in cancers and other diseases among smokers is due in large part to the many non-nicotine substances found in cigarette smoke¹.

The smoker's habit is very complex and is associated with many factors. These include flavour, smoke, personality, mood and the social setting one happens to be in, which may involve having an alcoholic drink, being under stress or, among other things, having fun. Perhaps most important, however, is the rapid rise in blood levels of nicotine associated with inhalation of tobacco smoke². Indeed, an important

characteristic of all drugs that produce dependency is the time between behavioural administration (smoking a cigarette) and the drug's entry into the brain.

The US Food and Drug Administration has recently approved the over-the-counter sale of transdermal nicotine as a smoking cessation aid, a drug delivery system designed to avoid the harmful chemicals found in cigarette smoke. The slower absorption of nicotine offered by the transdermal nicotine patch relative to tobacco products should substantially reduce the likelihood of nicotine dependence in users of the patch. For example, a recent double-blind, placebo-controlled study investigating the therapeutic potential of the transdermal nicotine patch for ulcerative colitis found little evidence for nicotine withdrawal symptoms following discontinuation of the patch, despite 26 weeks of daily applications of 15-mg nicotine patches³.

Cigarette smoking is clearly a major health problem, and decreasing its use is a worldwide effort, but there is also a growing body of evidence to indicate that nicotine has therapeutic properties. Nicotine improves cognitive function and may reduce the symptoms of certain neuropsychiatric disorders such as Tourette's syndrome and attention deficit/hyperactivity disorder^{6,7}. But many of these efforts will be hampered if the role of nicotine in cigarette smoking is overemphasized without acknowledging the

essential differences between nicotine, tobacco and other drugs of abuse.

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Strange characters

SIR — J. T. C. Sellick (*Nature* **383**, 569; 1996) raises the issue of what a researcher has to do when he/she changes his/her name. The answer is simply to start using your newly acquired name.

There are some more important name issues, for example authors with names contain non-ASCII letters. We often see that our names change to accommodate the country we happen to live in. Many variations can therefore be found on various databases; a standard is not going to happen until we all speak the same language.

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Uppsala, Sweden. (And the rest of the world)

First, teach the teachers

SIR — When I started teaching, I thought teachers of English were to blame for poor scientific writing. Forty years later, I believe the main blame lies with teachers of science.

Much of what is said in lectures goes into students' notes without alteration of wording. Indeed, some teachers still write chunks of prose on the blackboard or on overhead transparencies, to ensure that students *get it down correctly* (my italics). I do not wish to debate whether that is good lecturing technique. My point is that lecturers' linguistic packaging of their subject-matter is something that students are likely to imitate and sometimes are obliged to copy.

The written items that accompany lectures are especially powerful influences. Handouts, example sheets, annotations on slides or charts — all these apparently represent the teacher's judgement of good ways to marshal and encode the material being studied. They are obvious sources of verbatim borrowings. Those borrowings become the currency of exchange in tutorial discussions, essays and examination answers. They begin to influence the mental set and the patterns of expression adopted by students.

If the models of accuracy, coherence and readability presented by teachers are good, the language training they give by example is beneficial. If the models they present are poor, their effect is pernicious. If the teachers themselves have little understanding of how language works, and of how to write readably, they are ill-equipped to evaluate students' writing and to show how that writing might be improved.

We have research-based knowledge of how to present scientific information accurately, readably and in styles that scientists themselves prefer to read. Unfortunately, many science teachers either are unaware of that knowledge or ignore it. Instead, they cling to the superstitions encapsulated by Simon Leather's letter "The case for the passive voice" (*Nature* 381, 467; 1996). Those superstitions are immensely influential. Time and again, young scientists, including young university teachers, have told me that they accept the findings of research into the readability of scientific writing, but that they dare not depart from 'traditional' passive, impersonal style for fear of their work being rejected by journal editors and by Leatherian academic supervisors. Given the points-scoring procedures that now affect survival in British universities, that fear is legitimate and serious.

How can we improve the situation? First, journal editors must remove the mythology that surrounds what they will and will not accept. A valuable step would be for all editors to stress that their Notes for Authors specify structure and format but not 'traditional' style. I do not know of any Notes for

Authors that specify or even recommend third-person, passive-voice writing for scientific papers.

Second, university science departments should jointly establish a programme in which staff could explore the research evidence on how to express scientific information accurately and readably. We need a joint initiative from senior academics, preferably supported by scientists in industry, to show new recruits that Leatherian views are *not* the views of the majority of the scientific community. Only when they are confident of that fact will young scientists, including university teachers, risk using and teaching anything other than 'traditional' turgid style.

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All the lab's a stage

SIR — John Emsley observes, in his review of the play *Blinded by the Sun* (*Nature* 383, 312; 1996), that during the past half century chemistry "has been almost ignored by writers and dramatists". Perhaps, but at least one offering merits mention.

The author is the Columbia University biochemist Erwin Chargaff, discoverer of the nucleotide-binding rules that bear his name — that adenine binds to thymine, and guanine to cytosine. The play (*Amphisbaena*, in *Voices in the Labyrinth: Nature, Man and Science*, by Erwin Chargaff; Seabury Press, New York, 1977) takes the form of a dialogue between an Old Chemist (transparently Chargaff himself) and a Young Biologist (James D. Watson, co-discoverer of the double-stranded helical structure of DNA) on the tension between chemistry and biology, and on the nature of scientific revolutions.

The dialogue is deliciously mordant, with such lines as "molecular biology is essentially the practice of biochemistry without a licence" and "life is what's lost in the test tube". And on the subject of some researchers winning and others losing in the quest for scientific immortality, the author concludes that "to make a scientific revolution one must break many eggheads".

Amphisbaena is still a timely commentary — and a good read.

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Divided by a common language

SIR — It is true that searching for data in bibliographic databases is greatly hampered by the differences between US and British spelling. It is also true that this problem will cause increasing problems in the future.

Koreaki Ito, in proposing the adoption of a universal spelling, correctly states that this would imply abandoning either British or American spelling (*Nature* 382, 666; 1996). He prefers the former option because he considers it more feasible, on the basis of figures that he does not detail. Is that because Japan (where Ito lives) is more oriented towards the United States than to Britain? Possibly so, although Ito probably does not intend to discriminate against Britain. I nevertheless dispute his preference for US spelling.

As a professional scientific editor, I find that British authors do, as a rule, object if their British English is changed into American English. Americans, on the other hand, rarely object if their texts are adapted to conform to British English. This may be an emotional reason to adhere to the British English spelling.

More important, however, is my observation that spelling in American English seems to be less consistent than in British spelling. Not only do American authors as a group spell words in different ways, but an American author may write the same word differently even within one manuscript. Opting for American English will not rule out the problem of different spellings, which is Ito's objective. It may be true that some spelling differences also exist in British English, but these differences are almost negligible when compared to the differences that can be found in American texts.

If science should opt for a universal English spelling — and I agree with Ito that it should do so as soon as possible — experts in each specific discipline should be consulted, as well as language experts. Many more spelling problems exist than Ito may be aware of. He mentions 'disulfide' and 'disulphide' to exemplify the problem: I have found, in one of the numerous chemical databases, for one single compound, the following terms: SO₂, sulfur dioxide, sulphur dioxide, sulfur dioxide and sulphur dioxide.

Eliminating such sources of confusion is most important. It would be necessary also to try to rule out synonyms, which pose a comparable problem when searching a bibliographic database.

A task for the International Council of Scientific Unions (ICSU), perhaps?

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The earliest memories of life on Earth

John M. Hayes

How old is life on Earth? Its chemical traces may have been recognized in rocks from Greenland that are more than 3.8 billion years old, at least 300 million years older than any previous evidence.

ITSQAQ is Greenlandic for 'ancient thing'. The Itsaq Gneiss Complex, a geological region in southern West Greenland, is indeed ancient¹. And, importantly, it contains the metamorphosed remains of the Earth's oldest-known sediments, providing evidence that there was something like an ocean 3.85 billion years (Gyr) ago. Until now it has seemed that these sediments might be relics of a prebiotic world, but the article on page 55 of this issue² may well remove that option. Specifically, new analyses of the traces of organic carbon present in the sediments change our estimates of its $^{13}\text{C}/^{12}\text{C}$ ratio. The new value is within the range characteristic of biological debris from sediments 2.7 to 3.5 Gyr old.

In modern oceans, both carbonates and organic molecules are derived from CO_2 through precipitation and photosynthesis. Isotopic effects associated with these reaction pathways lead to an isotopic difference, or fractionation, of about 25 parts per thousand, with the organic materials being depleted in ^{13}C relative to the carbonates. A similar isotopic signal persists, with some variations³, back through time to rocks that are 3.5 Gyr old.

The new finding seems to extend that record to the very bottom of our planet's sedimentary pile, crucially altering earlier views of these oldest sediments and leaving almost no time between the end of the 'late heavy bombardment' of bodies within the inner Solar System by giant meteorites and the first appearance of life. Two earlier papers published in this journal^{4,5} conclude that these asteroid impacts might have sterilized the planet as recently as 3.8 Gyr ago. It could follow that the 3.85-Gyr-old Itsaq organic material derives from biochemical processes that developed with breathtaking rapidity after the last large impact, or even that it is a product of a biota that was wiped out, then supplanted by our ancestors. Can this all be true?

Isotopic fractionations result from physical phenomena associated with chemical reactions. The task now is to determine whether the observed fractionations are due to biochemical processes, other low-temperature chemical reactions, or some post-depositional effect. The magnitude of the observed fractionations favours the biochemical alternative,

but does not fit perfectly. About half of the new analyses indicate fractionations larger than any in the next-oldest organic material, found in Australian rocks about 3.5 Gyr old, which also contain bacterium-like bodies and laminated sedimentary structures that resemble algal mats. These features are not considered a sure sign of life, but the carbon-isotope differences are roughly equal to those in

All previous analyses of such ancient materials have dealt only with bulk organic carbon, which shows all too well the hazards of an early birth. Any sediment that sits around for long is likely to be buried by still further sedimentation and then dragged into the crust's tectonic traffic. The Itsaq rocks were churned downwards within a billion years and heated to 500 °C at pressures of 5,000 at-



C. R. L. Friend

A chip off the old block. A geologist's hammer leans against the oldest-known sediments in the world, on the bleak Akilia island off the Greenland coast. Despite metamorphism, they show signs of ancient life.

still younger rocks that have compelling evidence for biology, so many investigators have proposed that life was present 3.5 Gyr ago and that it was controlling the redox chemistry of carbon at Earth's surface.

The new Itsaq data were gathered using an ion-microprobe mass spectrometer that can selectively analyse features as small as 20 μm . That selectivity has been used to examine carbon occluded in apatite grains (see Fig. 1 on page 56). Apatite, a phosphate mineral, is known for its biological associations, but there have been no previous measurements of the isotopic composition of organic carbon closely associated with it. Interpretations of the new data may evolve as points of comparison become available. Although the age of the samples and the tantalizing results command interest and prominent publication, the novelty should also inspire caution.

mospheres. From the point of view of anyone seeking to reconstruct details of their initial, low-temperature environment, they've been badly overcooked. Although some previously analysed samples contain as much as 2.5 per cent carbon⁶, it looked more like graphite than biological debris.

During graphitization, mobile chemical products wheeze or ooze from the geological pressure cooker wherever a vent can be found. If carbonate minerals are present, their carbon is thrown into the chemical m  le. In the process, isotopic evidence is first altered (as some carbon depleted in ^{13}C is lost) then destroyed (as the residue exchanges carbon with metamorphic fluids^{7,8}). For this reason, results of earlier analyses of the Itsaq materials were set aside as they were received, because they showed the damage done by graphitization. When the authors examined bulk rather than apatite-

associated carbon, their results agreed with those of previous analyses.

What is missing is an understanding of why the apatite should act as a safe-deposit vault. Until that is known, other questions will claim attention. First, why are the $^{13}\text{C}/^{12}\text{C}$ ratios so variable and, in half of the cases, so low? Biological fractionation is usually very consistent, and ratios like those in the lower half of the present distribution do not appear for more than a billion years, when they are thought to be produced by methane-consuming bacteria. These organisms require oxygen, which fits well with other indicators of increasing oxygenation 2.7 Gyr ago, but not 3.85 Gyr ago. Second, is there a plausible non-biological cause for the fractionation, either an isotope effect associated with one of the infinite number of uninvestigated prebiotic chemical reactions, or some obscure post-depositional effect peculiar to microscopic inclusions in apatite? These are worries and long-shots. It's most likely that the new results are indeed evidence for life 3.85 billion years ago.

The use of an ion microprobe to measure carbon isotopes is new. The probe works by firing caesium-ion bullets into a polished chip to break up crystals and molecules at its surface. Negative ions (C^- in this case) are extracted and accelerated, and the isotopes are electromagnetically separated by a mass spectrometer. It is assumed that any instrumental effects biasing the ratio observed from the thin films of carbon within the mineral matrices can be removed by comparison with a reference sample of pure graphite. Tests indicating consistency with results of conventional analyses are impressive, but more documentation of the probe's suitability for carbon-isotope analyses will be welcomed. As soon as that is available, curators of collections of martian meteorites and of ancient microfossils should start getting in line. □

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X-ray lenses near reality

Jerome Hastings

X-RAY sources have already evolved from the original sealed vacuum tubes into sophisticated electron-storage rings, and free-electron X-ray lasers may be just beyond the horizon. These advances promise intense, highly parallel and tuneable photon beams. To focus and manipulate this radiation, a variety of X-ray optical devices have been developed, ranging from concentrators, in the form of tapered capillaries¹, to Fresnel zone plates². But a dream of X-ray opticians for some time has been to make a *refractive*

beams prohibits such applications.

The existing optics fall into a few categories: reflecting optics such as grazing-incidence X-ray mirrors; tapered capillaries; and various types of Fresnel zone plates. Refractive lenses, routinely used in visible-light optics, had been thought to be impractical at X-ray wavelengths. In the X-ray regime, the difference in the index of refraction between air and ordinary materials is tiny — typically of the order of 10^{-6} — which appeared to present an insurmountable problem. To maximize the effect, heavy-element materials were proposed, but they have high absorption. Light elements do not, but with their low refractive-index decrement they would have long focal length and thus provide little practical focusing of the X-ray source.

ESRF

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FOR COPYRIGHT
REASONS

The European Synchrotron Radiation Facility in Grenoble, France. Compound refractive lenses may soon make its intense X-ray beams much more effective.

lens, the X-ray equivalent of conventional optics for visible light. On page 49 of this issue³, Anatoly Snigirev and colleagues describe the first significant step towards that goal. This should allow us to reach a submicrometre focus with much greater efficiency than before.

The desire in a multitude of scientific disciplines for intense submicrometre X-ray beams has driven the construction of new electron-storage-ring X-ray sources in Europe (the European Synchrotron Radiation Facility in Grenoble, France), the United States (the Advanced Photon Source in Argonne, Illinois) and Japan (the Spring-8 Project in Harima). The objective is to perform the various 'standard' X-ray analytical techniques of diffraction and spectroscopy on individual particles or grains.

These measurements could provide information about the atomic and electronic structure of systems of chemical, biological and technological interest, ranging from the characterization of an individual grain in a commercial catalyst to measuring the strain in the metallic interconnections of large integrated circuits. But the limited ability of available X-ray optics to focus the X-ray

Snigirev and colleagues³ have now proposed and demonstrated a compound refractive lens for hard (14 keV) X-rays that overcomes the difficulties of earlier schemes. A series of N refractive lenses has a focal length shorter than a single lens by a factor of N , and, in aluminium, absorption is slight for X-ray

wavelengths below one ångström, so a series of tens to hundreds of lenses can be used. The authors made their lens by simply drilling small round holes in an aluminium block (see Figs 1 and 3 on pages 49 and 50, respectively).

This 'crude' technology permitted a demonstration of their ideas and showed the potential for future optics. With more sophisticated fabrication techniques, one can imagine extending these lenses from cylinders to spheres to provide focusing in two dimensions. Such lenses should then be commonplace at synchrotron sources, and the full potential of the X-ray beams already available will be realized. This development bodes well for the future of synchrotron-based microanalytical techniques that are impossible today but should be standard in the future. □

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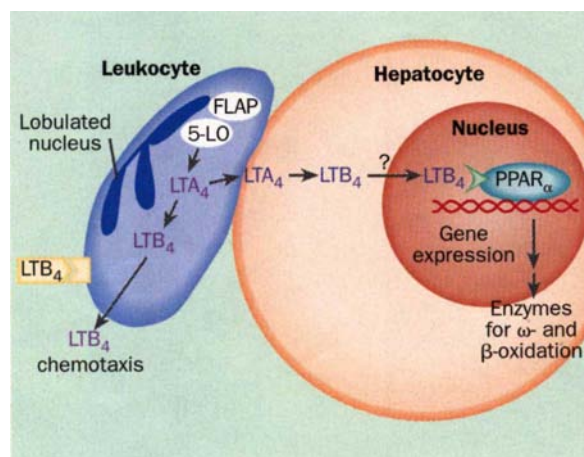
Charles N. Serhan

Eicosanoids are synthesized from arachidonic acid, which is a membrane-derived fatty acid. The enzymes that catalyse this reaction, the cyclooxygenases and lipoxygenases, either translocate to or reside in the nucleus (Fig. 1). For example,

The precursors to LTB_4 are 5-hydro-

```
graph TD; A[Novel anti-inflammatory agents] --> D[PPARα]; B[LTB4] --> D; C[Hypolipidaemic drug Wy14,643] --> D; D --> E[Transcription factors, nuclear receptors]; E --> F[Inactivation of pro-inflammatory mediators]; E --> G[Obesity Control of overall lipid metabolism]; F --> H[Inflammation Control of duration and extent]; E -.-> I[?]; I -.-> J[Specific eicosanoids and other lipid mediators?]; J --> G;
```

How can these findings help us to understand the interactions between the lipid-mediator networks and transcription, and how might they influence drug design? A model for the possible interactions is shown in Fig. 2, and an intriguing aspect of it is whether the non-steroidal anti-inflammatory drugs and the newer, cyclooxygenase and lipoxygenase inhibitors¹ have an impact on the control of overall lipid metabolism — that is, obesity. Conversely, do agents that lower the amount of lipid in cells affect inflammation? Also, do specific members of other classes of eicosanoids, such as the thromboxanes, epoxyeicosatetraenoic acids or aspirin-triggered 15-epi-lipoxins, activate transcription factors? Thrombox-



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ane and prostacyclin have opposing functions in haemodynamics; and leukotrienes are pro-inflammatory, whereas lipoxins seem to act as endogenous anti-inflammatory eicosanoids¹. So it will be of interest to learn whether these groups of eicosanoids also have opposing effects on the transcriptional machinery.

Devchand *et al.*² have also shown that a hypolipidaemic drug that lowers triglyceride levels can activate PPAR α . This hitherto unknown mechanism of activation offers a molecular model for rational drug design, to synthesize selective lipid-lowering agents as well as potential anti-inflammatory agents (Fig. 2). It is likely that the mechanism by which eicosanoids bind to PPARs and activate transcription involves conformational changes in the ligand-receptor complexes that dictate the sites of interaction with target genes. It follows that the binding of individual eicosanoids to PPAR α may yield unique ligand-PPAR α complexes that lead to differential gene expression. Along these lines, several leukotriene-receptor antagonists have been developed that very effectively block LTB₄ binding and function, although the clinical effects of these agents are not yet known⁷.

Given the findings of Devchand *et al.*², it may be worth screening existing synthetic libraries of LTB₄ neutrophil antagonists and mimetics which have structural similarities to LTB₄, to test for their ability to activate PPAR α and to lower the levels of triglycerides. Certain hypolipidaemic drugs may also have an anti-inflammatory action, and it should be possible to design anti-inflammatory agents that increase PPAR α , allowing the increased expression of particular genes. This new link between membrane-derived bioactive lipids and gene expression opens up many opportunities for investigations into cell signalling and new targets for drug design. □

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A new wave for heart rhythms

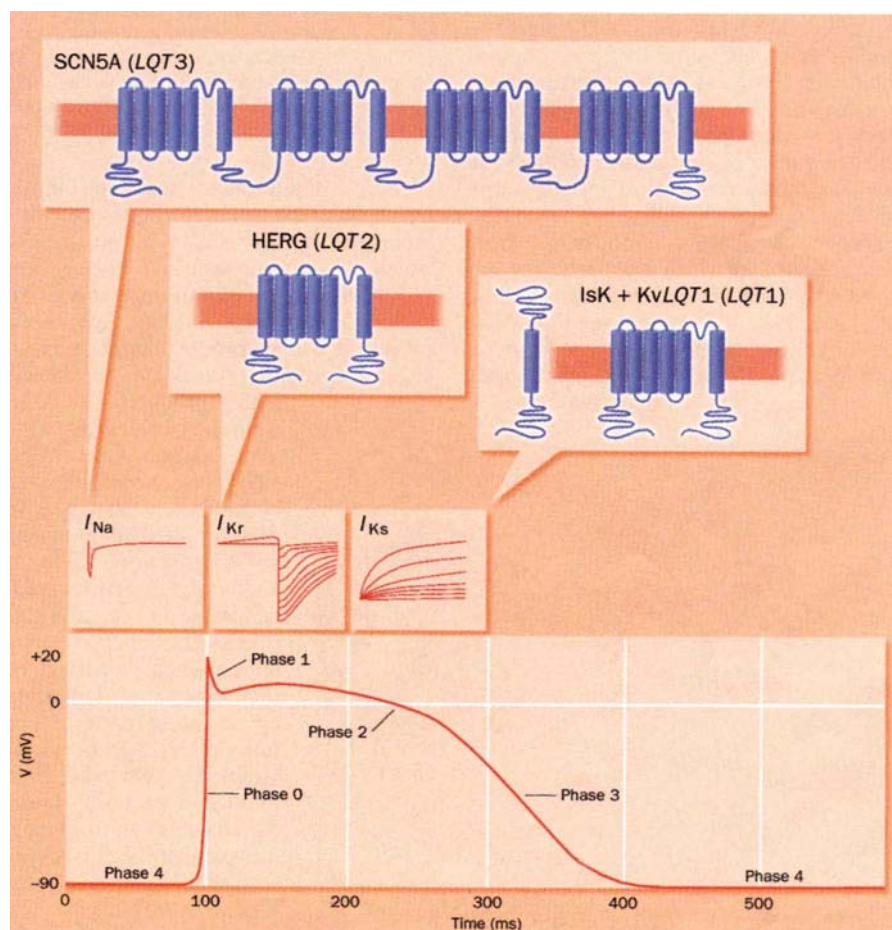
Bernard Attali

THE plasma membranes of cardiac muscle cells contain a complex repertoire of voltage-gated ion channels that are responsible for the generation of action potentials — travelling waves of electrical excitation. The delayed-rectifier K⁺ channels are involved specifically in the repolarization of cardiac-cell membranes following an action potential, and abnormal behaviour of these channels is thought to cause deviations from the normal rhythms of the heart, so-called arrhythmias. Now, on pages 78 and 80 of this issue, Barhanin *et al.*¹ and Sanguinetti *et al.*² identify the molecular components of the slow delayed-rectifier K⁺ channels, a crucial step in the search for new anti-arrhythmic drugs.

In many species, including humans, the K⁺ current that passes through the cardiac

delayed-rectifier channels corresponds to the sum of two distinct currents: a rapidly activating current, I_{Kr} , and a slowly activating current, I_{Ks} . Dysfunction of these channels causes the long-QT syndrome (LQT), an inherited disorder that increases the risk of sudden death from cardiac arrhythmias^{3,4}. The I_{Kr} voltage-gated K⁺ channel is the product of the *HERG* gene, mutations in which map to the *LQT2* locus³. Now I_{Ks} has also revealed some of its molecular secrets^{1,2}. The new results show that the association of two structurally different membrane proteins, K_vLQT1 and IsK (minK), reconstitutes the cardiac slow delayed-rectifier I_{Ks} current.

The K_vLQT1 protein is a newcomer to the LQT club, and it was identified by positional cloning to the *LQT1* locus⁴. The



Following an action potential, mammalian cardiac cells are repolarized by the activation of a variety of K⁺ channels. The molecular components of the channel that underlies the slowly activating current I_{Ks} have now been identified by Barhanin *et al.*¹ and Sanguinetti *et al.*². The *LQT1* locus encodes the K_vLQT1 channel, which associates with IsK protein to form I_{Ks} . The *LQT2* locus encodes the HERG K⁺ channel, which underlies the rapidly activating current I_{Kr} . The outcome from the two channels is a small net outward current that increases with time, and mutations in *LQT1* and *LQT2* lead to a loss of channel function. The *LQT3* locus encodes the cardiac Na⁺ channel, SCN5A, and mutations in *LQT3* lead to a gain of channel function. Despite their differences, mutations in K_vLQT1, HERG and SCN5A all lead to the long-QT syndrome, an inherited disorder characterized by delayed repolarization and cardiac arrhythmia.

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partially cloned complementary DNA revealed that the *KVLQT1* gene encodes a hitherto undiscovered K^+ channel that is structurally similar to voltage-gated K^+ channels — it has a conserved K^+ -selective pore-signature sequence flanked by six putative membrane-spanning segments. By contrast, IsK is a small, type-III glycoprotein that has a single putative membrane-spanning segment but neither sequence nor structural homology with any other cloned channels⁵ (see figure). However, there is a remarkable correlation between the distribution of *KVLQT1* and *IsK* gene transcripts in heart, pancreas, kidney and salivary gland. This was, perhaps, the hint for the clever connection.

The *KVLQT1* complementary DNA has now been fully cloned and expressed in various heterologous systems^{1,2}. Curiously, the expression of K_vLQT1 alone induces a voltage-dependent K^+ outward current that has very different biophysical characteristics from the native K^+ currents recorded in ventricular cells. For example, the K_vLQT1 -activating kinetics are much faster than those of I_{Ks} and, in contrast to I_{Ks} , no inward rectification is apparent at positive potentials. However, with the exception of *Xenopus* oocytes, the expression of IsK alone does not produce detectable K^+ -channel activity in transfected cells.

The breakthrough has been made by the coexpression of K_vLQT1 and IsK. This induces a slowly activating delayed-rectifier K^+ current that has similar gating kinetics to the native I_{Ks} , but a much higher amplitude than that observed with K_vLQT1 alone. Moreover, there is a +20-mV rightward shift in the voltage-dependence of activation when K_vLQT1 and IsK are coexpressed. Clearly, the biophysical properties of the K_vLQT1 /IsK complex match those of the native I_{Ks} whereas those of K_vLQT1 alone do not.

To reach these conclusions, Barhanin *et al.*¹ used immunoprecipitation to demonstrate the physical interaction between K_vLQT1 and IsK, and Sanguinetti *et al.*² cloned the *Xenopus* analogue of K_vLQT1 . These compelling data settle a long-running debate on two issues^{6,7}. First, IsK alone is unable to form a functional K^+ channel. And second, IsK induces K^+ currents in *Xenopus* oocytes through interactions with endogenous K_vLQT1 channels. In a way, K^+ channels are more conformist than we expected, because they need a K^+ -selective pore-signature sequence.

The clinical implications of these findings are numerous. The cardiac I_{Ks} channel is a target of class-III anti-arrhythmic drugs, and blocking it can delay repolarization and induce an acquired form of LQT. On the other hand, positive regulation of I_{Ks} by drugs such as fenamates might be helpful⁸. In either case, a sharp picture of the molecular contours of the K_vLQT1 /IsK channel complex should provide clues for drug design. The status of K_vLQT1 and IsK should also be examined in the autosomal recessive form of LQT, known as Jervell-Lange-Nielsen syndrome, which is associated with congenital neural deafness. A careful study of the effects of *KVLQT1* mutations on channel function and their correlation with clinical symptoms should also be informative. Like mutations in the *HERG* gene, the *KVLQT1* mutations may act in a dominant-negative fashion and lead to a loss of channel function.

The biophysical impact of these stimu-

lating data raise many questions that should stimulate innovative studies on channel gating, permeation, assembly and stoichiometry. By which 'unusual' mechanisms does IsK reduce the K_vLQT1 gating kinetics? To some extent, IsK bridges the activation of K_vLQT1 , but in so doing renders the K_vLQT1 channel more efficient. Does the IsK protein form part of the channel pore, and if so, what are the interacting domains? Single-channel studies should be very helpful in addressing this question. Finally, what are the interface domains and stoichiometries involved in the physical interaction between K_vLQT1 and IsK, and how are these important for channel assembly? We should soon be able to open our gates to a flurry of reports addressing these questions. □

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DNA REPAIR

Push and pull of base flipping

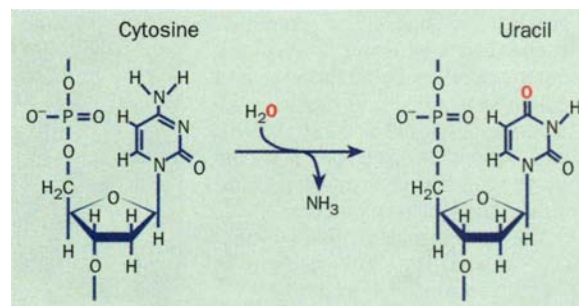
Thomas A. Kunkel and Samuel H. Wilson

ENZYMES that are involved in the modification or removal of specific bases from DNA — such as methyltransferases and glycosylases — processively scan the DNA¹ in their search for what are often rare binding sites. These exquisitely specific enzymes recognize and process their substrates by binding target bases outside the double helix^{2,3}. But we know very little about the mechanisms that could lead to such 'flipping' of bases to an extra-helical position by proteins that scan along a DNA molecule at rates approaching thousands of base pairs per second.

The report by Slupphaug *et al.*⁴ on page 87 adds significant new insight to this interesting problem. They present the crystal structure of human uracil-DNA glycosylase (UDG) bound to uracil-containing DNA. Their detailed view of the UDG active site suggests that the enzyme pushes the target uracil base out from the DNA helix and then pulls the base into its substrate-binding pocket where the uracil is excised.

In the next 24 hours, the DNA in every cell in your body will be spontaneously damaged more than 10,000 times⁵. To deal with this, and with the additional challenges to genomic integrity that arise from external insults (for example, ultraviolet light or chemicals), cells have several dif-

ferent enzymatic DNA-repair pathways. These include mismatch repair for correcting DNA-replication errors, and nucleotide-excision repair for correcting bulky adducts that often distort the helix and block DNA replication. Also included is base-excision repair⁶, which is initiated when DNA glycosylases recognize a variety of different modified bases and excise



Because cytosine normally pairs with guanine, cytosine deamination to uracil generates a G-U mismatch. If the uracil is not corrected by base-excision repair before the next round of replication, then adenine will be incorporated opposite the uracil, ultimately yielding a G-C to A-T transition.

them from the DNA. These bases arise as a result of the deamination, oxidation and alkylation reactions that frequently occur in cells during everyday metabolism. One of the most extensively studied base modifications is cytosine deamination, which yields hundreds of uracil bases per human cell per day (see figure). These are highly mutagenic if unrepaired, leading to G-C to A-T transitions. So, to remove uracil and initiate repair, cells express UDG. ▶

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Based on earlier studies of UDG-crystal structures in the absence of DNA⁷, Slupphaug *et al.*⁴ produced a mutant form of UDG that had a reduced catalytic efficiency and an increased affinity for DNA. The crystal structure of this enzyme bound to a 10-base-pair DNA duplex with a central U-G mismatch reveals that the UDG active-site groove interacts, for four consecutive nucleotides, with the DNA backbone of the strand in which the uracil is found. The backbone flanking the uracil is compressed, and the uracil, deoxyribose and 5' phosphate are flipped out of the helix into the major groove. Here they are stabilized by interactions within an enzyme pocket that defines specificity for the removal of uracil but not of the four normal bases. The flipped uracil is positioned in the active site and the N-C 1'-glycosylic bond is not observed — that is, this is an enzyme-product complex. In place of the nucleotide that has been displaced from the DNA helix is the side chain of the UDG residue 272 (normally a leucine, but an arginine in the mutant used here), which is inserted through the minor groove.

It has been proposed that base flipping to promote catalysis is common among a number of different DNA repair and base-modification enzymes^{2,3}. But these enzymes each present interesting variations on the flipping theme. *Escherichia*

coli photolyase is thought to flip out a cyclobutane pyrimidine dimer to allow the direct photoreversal of the dimer⁸. In contrast, when T4 endonuclease V removes dimers, it has been suggested that it doesn't flip the dimer itself, but rather the opposing undamaged 5' base. The flipped base produces a cavity in the DNA duplex that allows access to the dimer for the enzyme's catalytic residues⁹. It has also been proposed that T7 DNA ligase flips the AMP residue that it covalently links to the 5' phosphate of nicked duplex DNA. In this case, the flipping occurs to prevent interference of AMP with subsequent ligation¹⁰. And the structures of two cytosine methyltransferases bound to DNA also reveal flipped bases¹¹ that are methylated and then returned to the helix.

Structural data indicate that the processes by which these flipping enzymes recognize their substrate DNAs may also be different. Damaged DNA that is bound to T4 endonuclease V is sharply kinked at the central thymine dimer, and there is considerable deformation of the phosphate backbone⁹. Along with a dimer-induced conformational perturbation in the duplex DNA prior to binding, these features may define initial recognition. In the case of the sequence-specific methyltransferases¹¹, the base flips into the minor groove and the vacated space is occupied by resi-

dues that enter from the major groove.

Interestingly, just the opposite is true for UDG, which is not sequence specific. Residue 272 enters from the minor groove, and the flipped uracil occupies the UDG specificity pocket in the major groove⁴. Does this suggest that UDG, and possibly other repair glycosylases, initially recognize their substrates by scanning the minor groove for subtle differences from normal geometry? This proposal has already been made for another class of non-sequence-specific DNA-processing enzymes, the DNA polymerases. For example, it has been suggested that DNA polymerase- β , the polymerase used for base-excision repair, may probe the positions of hydrogen-bond donor and acceptor groups in the DNA minor groove to discriminate between correct and incorrect base pairs¹².

The extremely low catalytic rate of *HhaI* methyltransferase is consistent with the possibility that methyltransferases simply capture cytosines that spontaneously unstuck from their neighbours and flip into the minor groove of the helix^{3,11}. Based on the much higher catalytic rate of UDG and the new structure of the UDG-DNA complex⁴, Slupphaug *et al.* suggest that UDG actually facilitates base flipping into the major groove. It is thought that UDG uses certain amino acids to compress the DNA backbone and 'push' the uracil base out of the helix, while other amino acids 'pull' or stabilize uracil in the flipped-out configuration. This leads to an obvious question: does UDG flip out every base that it encounters and then capture only those that can be accommodated by the specificity pocket, or does it flip out only the base that it is designed to repair? The rich detail of the new UDG-DNA co-crystal structure provides exciting possibilities for experiments to address this and other questions on the mechanism of action of this exquisitely specific and critical DNA-repair enzyme. □

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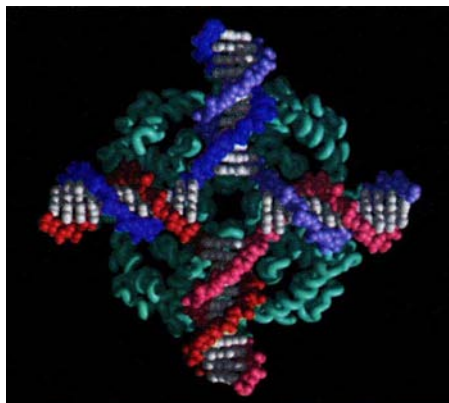
DNA RECOMBINATION

Four pins for four petals

DNA-binding proteins come in many shapes and sizes, reflecting the diversity of the DNA structures to which they attach. The crystal structure of one such protein — *Escherichia coli* RuvA — has now been solved by John Rafferty and co-workers (J. B. Rafferty *et al.* *Science* 274, 415–421; 1996), and they have proposed a model for the binding of RuvA to its substrate, the Holliday junction.

The four-stranded Holliday junction is a central intermediate in genetic recombination, and movement of the junction point allows the generation of heteroduplex DNA in a reaction that is driven by the RuvB DNA helicase. The functions of RuvA are to target RuvB to the Holliday junction and to hold the junction in a square planar configuration. And now the crystal structure reveals just how this is done.

RuvA protein is known to assemble into tetramers, and Rafferty *et al.* show that the monomers are related by fourfold rotational symmetry that is reminiscent of a four-petaled flower. Protruding from the concave face of each monomer is a small, negatively



charged pin, and when a model of the tetrameric structure (green) is superimposed onto a model of the Holliday junction (DNA backbones shown in purple and red), the four pins fit neatly through the hole at the centre of the Holliday junction. These pins encourage the transient separation of the DNA strands within each arm of the junction because of repulsive interactions between the pins and the phosphate backbones. In this way, RuvA holds the DNA in the requisite conformation for processing by the RuvB helicase.

Alison Mitchell

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The buzz about pollination

Peter D. Moore

A PUZZLE for pollination ecologists has been explaining the advantages of the structure of certain types of flower, such as some nightshades (including potato), which have reflexed petals, and stamens that form a projecting conical mass as shown in the photograph. One suggestion is that visiting bees cause pollen-shedding by buzz-pollination (sonication pollen-dispensing to the purist), so keeping the petals out of the way is a means of reducing their potential damping of the vibrations. But such a proposal leaves many questions unanswered — what, for instance, is the frequency required for pollen release, and how does the flower avoid shedding its entire load of pollen when stimulated?

These issues have been investigated by M. J. King and S. L. Buchmann¹, and their conclusions are reported in *Functional Ecology*. The key, they feel, is not in the buzz frequency, but in the force that the bees can exert on the pollen-containing heads of stamens, the anthers, by accelerating their vibrations and throwing the pollen out of the exit pore.

Buzz-pollination is an appealing but not strictly accurate term given to a system of pollen collecting (rather than pollen depositing, as implied by 'pollination') associated with certain bumble-bees (*Bombus* spp.). These bees are able to uncouple their wing movements from the actual mechanism of flight and can vibrate them, as when warming-up before flight and when communicating by sound². They may also perform this exercise when foraging from certain types of flower, including some of the nightshade family (Solanaceae), some Primulaceae, some heaths (Ericaceae), Boraginaceae and various others.

These flowers have several characteristics in common. They often provide a reward of only pollen for the visiting bee; they usually have bent-back petals revealing a conically arranged series of anthers (sometimes brightly coloured); and the anthers release their pollen through an apical pore, in a salt-cellar fashion. The flowers are also normally pendent and typically have very small pollen grains (less than 25 µm).

According to Barth³, buzz-pollination was first recorded in 1959 by John H. Barrett in the highlands of New Guinea, when he noticed low-frequency buzzing sounds coming from foraging bees in the forest, and it has subsequently been observed in many plants in virtually all climatic zones. Pollination ecology is a particularly fertile field for the study of co-evolution and the biophysics of buzz-pollination has presented some intriguing challenges. What

is the most effective frequency of vibration for pollen release? Does this correspond to the natural frequency of the flower? Is this the frequency operated by the visiting bee?

Working on the North American buzz-pollinated member of the Primulaceae, the shooting-star (*Dodecatheon conjugens*), Harder and Barclay⁴ found that vibrations of 400 Hz and below (the

IMAGE
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REASONS

Design for giving — the structure of the flower of the potato, *Solanum tuberosum*, lends itself to pollen-dispensation by sonication by visiting bees.

frequency used by bumble-bees) resulted in less than 10% of the available pollen being released, whereas higher frequencies liberated over 23% of the pollen. Their explanation of the difference was that the higher tuning frequency of the flower ensured that a visiting bee did not take away too high a proportion of the pollen — the equivalent, for the flower, of not placing all of one's eggs in one basket.

The story, however, has not proved so simple and some workers have found no relationship between frequency and pollen liberation; others have proposed that a gradual drying of the pollen may regulate the release⁵. In an attempt to clarify the situation, King and Buchmann¹ have turned to a New Zealand nightshade, the poroporo (*Solanum laciniatum*), and have examined not only frequency effects, but also the role of accelerating frequency in stimulating pollen shedding. Their argument is that, because force is equal to mass times acceleration, a low-frequency vibration with rapid acceleration can

generate a high pollen-releasing force.

Flowers that were ripe but had not shed their pollen were found to have natural frequencies of 195 Hz, which became lower following dehiscence of the anthers. Foraging *Bombus* bees were observed to apply buzz frequencies of around 300 Hz to the *Solanum* flowers and generated vibration accelerations of about 180 m s⁻¹, which were sufficient to cause pollen ejection (the minimum acceleration required was 105 m s⁻¹). So the force applied by the bee to this flower is adequate for the purpose and the plant is not economizing on pollen by being tuned to a higher frequency.

Heather Angel

The initial sonication, however, released only 18% of the available pollen, but this was still a relatively large load (about 72,000 pollen grains). Yet the flower still retains a significant proportion of its pollen production for future visitors. King and Buchmann suggest that this is being achieved by a gradual drying process within the tissues of the anther so that more pollen grains are made available for sonication at the next visit. This ensures that pollen is dispensed from a particular anther over a period of time, thus increasing the dispersal potential of the grains and the likelihood of success in widespread pollination. The bees are evidently aware of these limitations to the success of vibration-induced pollen harvesting because they commence their foraging with a low-magnitude buzz to check that the flower is in a position to respond to more intense sonication before further energy is expended on that activity.

The need for dryness in the pollen prior to sonication may also explain the recurved petals and the pendent habit of many buzz-pollinated flowers.

The efficiency of the buzz in persuading an anther to part with a proportion of its pollen load makes one wonder how widespread this phenomenon may be, especially among flowers equipped with anthers that open through a terminal pore. Giving it a quick shake seems such an obvious answer to many a bee's problems. □

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A new turn for Archimedes

David Hoffman

'OIL and water don't mix' says the old saw. But many immiscible liquids, in the presence of a soap or some other surfactant, can self-assemble into a rich variety of regular phases. Characterized by their inter-material dividing surfaces — where the different substances touch — these

ing, well known in the study of blue phases"^{1,2} (Fig. 1b).

The model surface with the first three properties is the helicoid — the surface swept out by a horizontal line rotating at a constant rate as it moves at constant speed up a vertical axis (Fig. 2). It divides space

curvature (another way to characterize minimality).

Why do such surfaces occur in nature? Reducing the surface area between two materials that are naturally repelling will reduce the total energy. It is therefore plausible that, to first order at least, their interface should be a minimal surface. Since that is true everywhere in the substance, it is also reasonable to expect that, at a supramolecular length scale, the structure should be homogeneous, that is, the interface should be periodic.

How would you recognize a periodic, space-dividing minimal surface if you happened to run into one? The helicoid was identified as a minimal surface in 1776, but the next periodic examples were not discovered until the 1830s, by Hermann Scherk (see ref. 3 for a description), and the first triply periodic example was found only some 35 years later by Hermann Schwarz^{4,5}. More examples were found around the turn of the century, but the subject slowly receded below the mathematical horizon. In fact, periodic minimal surfaces have gone in and out of fashion for the past 150 years.

The latest revival dates to the 1960s, when A. Schoen, then working for NASA and interested in strong-but-light structures, found several new triply

periodic, space-dividing minimal surfaces^{5,6}. For many years these surfaces were better known among materials scientists than among mathematicians. But since the early 1980s they have again been of interest to differential geometers, in part due to their importance in materials science. Recognizing minimal surfaces is much easier now that computer simulation and graphics are widely available (see ref. 7, for example).

Schoen's most spectacular discovery was the gyroid (Fig. 3a). After 30 years of obscurity, it is currently the darling of researchers who study block copolymers. Claims have been made that this surface and its companion constant-mean-

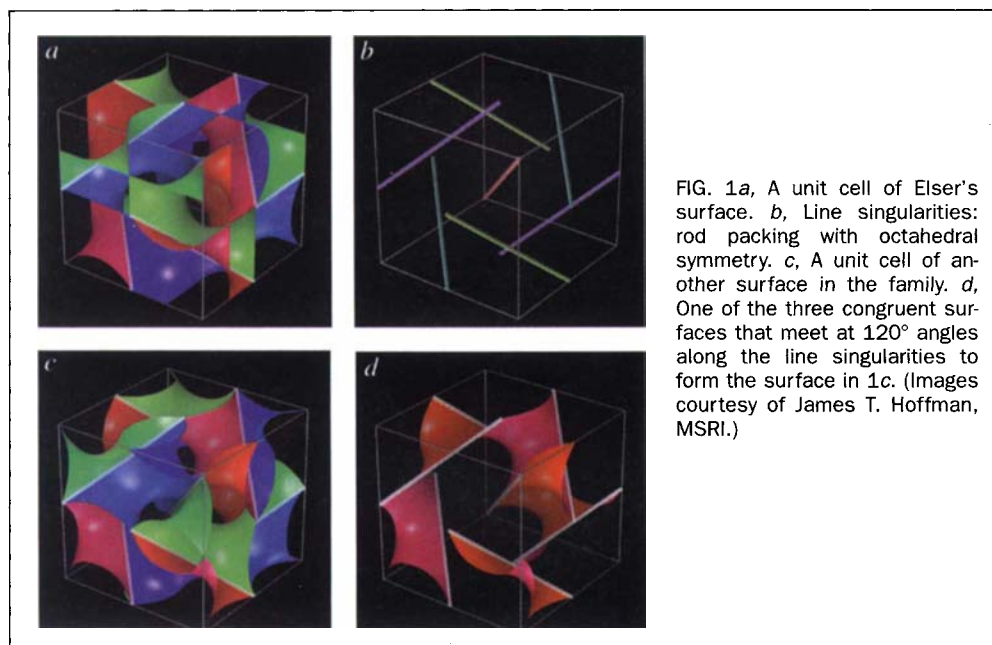


FIG. 1a, A unit cell of Elser's surface. b, Line singularities: rod packing with octahedral symmetry. c, A unit cell of another surface in the family. d, One of the three congruent surfaces that meet at 120° angles along the line singularities to form the surface in 1c. (Images courtesy of James T. Hoffman, MSRI.)

structures also occur in microphase-separated block copolymers. Understanding the interface is vital to the prediction of material properties, but at present the relationship between the curvature of the dividing surfaces and the relevant molecular and macromolecular physics is not well understood. Moreover, there is only a partial theoretical understanding of the range of possible periodic surfaces that might occur as interfaces. Here, differential geometry, the mathematics of curved surfaces and their generalizations, is playing an important role in the experimental physics of materials. A recent special issue of the *Philosophical Transactions of the Royal Society of London* provides a good sample of current work.

In one article called "A cubic Archimedean screw"¹, the physicist Veit Elser constructs a triply periodic surface with cubic symmetry (a unit translation in any one of the three coordinate directions moves the surface onto itself; Fig. 1). Motivated by investigations in materials science, and particularly the properties of blue phases of liquid crystals, Elser constructs his surface with a number of properties in mind: handedness (or 'chirality'), Archimedean-screw-like behaviour, and minimality. Also, the singularities of the surface are dictated by "the O⁸-rod pack-

into two congruent regions, and we can take the helicoid to be the boundary of either one of them. It is evident that a vertical translation has the same effect on the helicoid as a proportional rotation about the vertical axis. (In particular, translate enough to make one full rotation and you are back to the original surface. That shows that it is singly periodic.)

Put a helicoid into a vertical cylinder filled with fluid and you have an Archimedean screw, a rotation of which moves the fluid up or down. Which way the fluid is pushed depends on which way the generating line of the helicoid turns around the axis; so helicoids have handedness. And the helicoid is a minimal surface, a property important in materials science.

To understand what a minimal surface is, imagine the surface sculpted from a thin rigid material. Cut out a small piece, save it, and form a soap film over the hole. The shape of the film is determined by the boundary of the hole and the physical behaviour of the soap film, which tries to minimize its area. If the soap film matches the piece you saved, and if this works everywhere you try it, the surface is minimal. An engineer might think of a minimal surface as a membrane between two gases at the same pressure, which by the Laplace-Young law will have zero mean

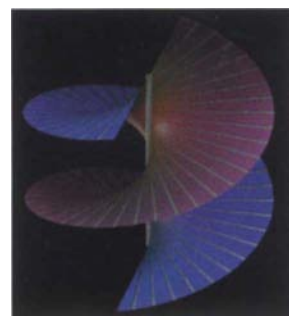


FIG. 2 The helicoid. (Image courtesy of James T. Hoffman, MSRI.)

Olbers' uproar

OLBERS' astronomical brightness paradox argues that if the Universe is infinite and evenly populated with stars, the night sky should be infinitely bright. Daedalus is applying an acoustic form of the argument to parties and social gatherings. Divide the area round any listener into annular shells. Shells of larger radius are more distant, but contain more people. Accordingly, with an acoustically reflective floor and ceiling, each shell will radiate to the central listener an added equal increment to the hubbub. An infinite party would therefore be totally deafening.

As with Olbers' paradox, there are several ways out. Sound has a finite speed, so sufficiently distant voices will not reach the listener until the party has been going on indefinitely long. Or the party-goers might form clusters which are themselves clustered, and so on, defeating the summation. The party might even be expanding.

Most parties, however, are even noisier than Olbers would predict. Each talker speaks ever louder to overcome the hubbub of the others, who react by speaking louder still. A further aggravating factor is background music. Initially it serves a useful purpose; it masks the occasional awkward silence when the few early arrivals all happen to fall silent together. Thereafter, it merely stokes the acoustic catastrophe.

Pop radio stations use a constant-level mixing system, which fades the music for the disc jockey's voice. In a restaurant or a party, such 'fill-in' music would mask the silences, yet fade towards zero intensity as the crowd noise rose. But to prevent acoustic catastrophe, further rises in noise level would require music of negative intensity.

So Daedalus is inventing it. A set of uncorrelated conversations contains all frequencies. Subtract the frequencies you don't want, and you could get any desired tone or musical chord. Daedalus's 'Negative Muzak' is created by a set of fast-acting, damped acoustic cavities around the room, which absorb a chosen programme of frequencies. In a roomful of talkers, they will extract just that sequence of frequencies from the hubbub which leaves behind the programmed tune. With luck, the perfect subtraction of just one frequency from white noise will imply that frequency to the ear; but for the strongest effect, more complete subtractions may be needed. Negative Muzak will provide background musical entertainment while subtracting from the acoustic hubbub rather than adding to it. Frustrating Olbers, it will bring welcome civility to restaurants and parties everywhere.

David Jones

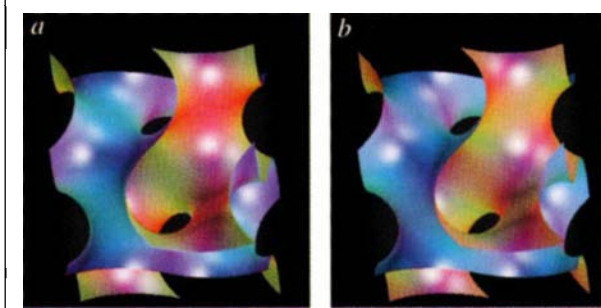


FIG. 3a, The gyroid, a triply periodic, space-dividing minimal surface that contains no lines and has no reflective symmetries. b, Almost identical, the solution of $\sin x \cos y + \sin y \cos z + \sin z \cos x = 0$. (Images courtesy of James T. Hoffman, MSRI.)

curvature surfaces are found as dividing surfaces in many materials⁸.

To simplify calculations, materials scientists and crystallographers have substituted for the gyroid — and for most other triply periodic minimal surfaces — the locus of solutions to a single trigonometric equation. For example, the solution to $\sin x \cos y + \sin y \cos z + \sin z \cos x = 0$ is, visually, amazingly close to the gyroid (Fig. 3b). Such 'zero-set surfaces' are useful in describing dividing surfaces where both materials have equal volume fraction; perturbations of them (with the zero replaced by a small constant) may be useful where the materials are unequal⁹.

These functions are not found by chance; they come from choosing an appropriate lowest-order term from the Fourier series of an electrostatic potential function coming from a charge distribution with the desired space-group symmetry. But there is as yet no explanation as to why this works, and in some cases it does not work very well. Moreover, these zero-set surfaces are not minimal surfaces, yet properties of minimal surfaces are claimed for them when convenient and ignored when they are troublesome.

Elser's surface¹ is the union of three zero-set surfaces. It is not minimal and there may not be a minimal surface close to it, in the sense that the gyroid is close to the zero set of the equation above. Moreover, the conversion of a rotational motion to a translation, the Archimedean-screw property, is not a property of this surface at all, but a property of a family of zero-set surfaces, considered as deformations of the original one (two examples are shown in Fig. 1). None of them are minimal and no two of them are congruent. They only have a weak form of handedness when taken as a family.

For a mathematician, this is troublesome. Consider that the gyroid has only recently been shown by rigorous mathematical means to be a space-dividing surface¹⁰. That is an important result, even though the evidence of its validity was already overwhelming from carefully generated models and computer images. For geometers, simulating a surface on a computer is a step along the way to understanding it mathematically, whereas a materials scientist has no use for an abstract surface without the ability to visualize it.

Archimedes had something relevant to say about this situation. Discussing the difference in mathematics between means of discovery and methods of proof, he wrote that "...certain things became clear to me by a mechanical method, although they had to be demonstrated by geometry afterwards because their investigation by the said method did not furnish an actual demonstration. But it is of course easier, when we have previously acquired, by the method, some knowledge of the questions, to supply the proof than it is to find it without any previous knowledge..."¹¹.

In thought-experiments and in real ones, Archimedes applied mechanics — the law of the lever in particular — to discover geometric relationships. He then tried to prove them by more formal means, and often he succeeded. What appears to be happening in materials science today can be viewed as an inversion of this process: new physical structures are being discovered by the sometimes very loose application of differential geometry. Their validation depends on whether these structures organize and predict observable phenomena, not on whether or not the theory was used correctly from a mathematical standpoint.

Materials science and mathematics may be immiscible, but with computer simulations and computer graphics as surfactant, they are interacting in unusual and productive ways. □

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Stuart Piggott (1910–96)

STUART Piggott was the last survivor of the "three wise men of British prehistory". Like the other two, Christopher Hawkes and Grahame Clark, he played a key role in the development of archaeology as a scientific discipline. Like them, he also had an international reputation for outstanding research and scholarly synthesis, which brought books on ancient India, prehistoric Europe, early wheeled vehicles, the history of antiquarian thought, and the Druids, to the shelves of scholars and the wider public. His mastery of the graphic arts and the written word brought an elegance to his work that made his research appealing to specialist and non-specialist alike; and his ability to convey the excitement and challenge of studying the distant past led generations of Edinburgh students to make their careers in professional archaeology.

Piggott knew from an early age that he was destined to become an archaeologist. He was publishing academic papers in the new international journal *Antiquity* from its first issue in 1927. A post in Reading Museum at the age of 17 was quickly followed by an appointment with the Welsh Royal Commission on Ancient Monuments; there he carried out excavations on prehistoric sites in Wessex, and began to publish papers on Neolithic sites and pottery which were to be the foundation for his important book, *Neolithic Cultures of the British Isles* (1954).

He was soon drawn to the interdisciplinary studies of the new archaeology, which was developing relationships with the natural sciences and anthropology, and as a result he was made a member of the Fenland Research Committee and Secretary of the British Bronze Implement Project in the early 1930s, and worked with the Stone Implement Petrological Survey from its inception in 1936.

In 1935 he helped found The Prehistoric Society, in whose first volume of *Proceedings* he published four papers. By 1936, the year he obtained the diploma of the Institute of Archaeology, he was working as assistant director with Alexander Keiller on excavations in Wessex, most particularly at Avebury (in Wiltshire, England), and he published one of his most influential papers on the 'Wessex Culture' in 1938. In it he examined a series of wealthy Bronze-Age burials, linking their contents to material from Ireland, central Europe and Mycenae, and characterizing their incumbents as princely traders — middlemen who controlled the trade between metalliferous Cornwall and continental Europe.

Work in the Central Air Photographic Interpretation Unit early in the Second World War did not impede his fieldwork, often undertaken with his archaeologist wife Peggy (later Peggy Guido), and led to his posting to a similar establishment in India, eventually to command military air photography in South-East Asia. There he rapidly taught himself the local archaeology, which resulted in a number of papers

ologically unacceptable', he was characterized as rejecting the dating technique altogether. But that was not his position, as the note makes clear, and he soon accepted the necessary changes to the chronologies, as did his contemporaries.

From the 1950s to the 1970s he was involved in excavations in Wessex (at Stonehenge, West Kennet and Wayland's Smithy chambered tombs) and Scotland (at Cairnpapple Hill, Croft Moraig stone circle and the Dalladies long barrow). He always viewed British prehistory in its European context, and his teaching at Edinburgh resulted in one of his most important books, *Ancient Europe* (1965), a brilliant synthesis of material from visits to western, central and eastern Europe.

As a boy, Piggott watched traditional wagon-building, and attributed his sympathetic understanding of pre-industrial technology to "a country upbringing when agriculture was virtually unmechanized". Seventy years later, he returned in his last book, *Wagon, Chariot and Carriage* (1992), to this abiding interest, but with the perspective of an exceptional international scholar whose knowledge of early wheeled transport and its construction was unequalled. His magisterial *The Earliest Wheeled Transport* (1983) drew on detailed knowledge of archaeological material from Eurasia and the Near East to examine the conflicting hypotheses for the development of wheeled vehicles in Europe, and favoured the rapid spread of a single invention over independent invention in more than one place. His research in this area was but one interest in a scholarly career that lay at the heart of the development of the science of archaeology.

Piggott's love of the Wessex countryside was a constant feature throughout his life, and it was the fertile archaeological ground from which grew many of his most significant contributions to the discipline. His expression of this relationship in 'Wessex Harvest', a poem in his collection *Fire Among the Ruins* (1948), showed another facet of this extraordinary yet self-effacing, brilliant yet approachable scholar, whose pleasure in good food, good company and good archaeology earned him the affection and respect of his colleagues, his students and the wider public from Uffington to the Caucasus.

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REASONS

and two books, *Some Ancient Cities of India* (1945) and the major synthesis, *Prehistoric India* (1950); it was the beginning of his impact on international archaeology. Returning to the United Kingdom as a Lieutenant-Colonel, he entered St John's College, Oxford, in 1945 for a B.Litt. on the antiquary William Stukeley; but before he had submitted the thesis, he was appointed to the Abercromby Chair of Archaeology at the University of Edinburgh in 1946, which he held until his retirement in 1977.

Piggott's publication in 1954 of *Neolithic Cultures* took place at a time when the 'radiocarbon revolution' had just begun. This dating technique, pioneered by Willard Libby in 1950, was to dramatically alter archaeological chronologies, and undo or invert some of the presumed cultural relationships between northwestern Europe and the Mediterranean. It had profound effects on the dating of the British Neolithic period and on Piggott's new synthesis, pushing dates back by up to 2,000 years, though much of his archaeological argument remained intact and the book still has considerable value today. Following a note in *Antiquity* in 1959, where he claimed that a radiocarbon date for Durrington henge was 'archae-

Competition for royalty in bees

SIR — Reproductive dominance is usually linked to morphological caste in honeybees, *Apis mellifera*, with the queen as the reproductive and the workers as the infertile individuals. However, in queenless colonies, workers can establish themselves as pseudoqueens, expressing all features of reproductive physiology that are otherwise typical of the morphological queen caste.

Pseudoqueens and sterile workers are completely different physiological units. In contrast to the sterile worker, the pseudoqueen has well developed ovaries, releases queen pheromones, elicits retinue behaviour, and suppresses fertility of other workers¹. Pseudoqueen behaviour is particularly well expressed in laying workers of the cape honeybee, *Apis mellifera capensis*. Within a few days after queen loss, an *A. m. capensis* worker can develop to a fully functional pseudoqueen². This physiological caste determination seems to be affected by genetic variance^{3,4}. Because the honeybee queen is highly polyandrous⁵, intracolony

genotype selection among subfamilies and individuals governs which workers are to become pseudoqueens and which remain sterile.

We studied the genotypic composition of four queenless splits of each of two *A. m. capensis* colonies over a 9-week period using single locus DNA fingerprinting⁶ (Fig. 1). Most of the patrilineal disappeared in the brood after dequeening, and workers of only a few patrilineal were able to produce offspring. Interestingly, the same subfamilies of a colony appeared to become reproductively dominant in all splits (Fig. 2). Using a sib analysis⁷ on the entire dataset, we estimated that 67% of the observed variance for reproductive dominance was due to between-family effects.

The high genetic variability of characters closely linked to fitness may seem surprising. However, a frequency-dependent selection model⁸ invoking both colony level and individual selection can plausibly explain the phenomenon. Colonies composed of dominant workers

alone are unable to produce any fertile offspring because there are no subordinate workers to attend to the brood or to rear adults. A majority of subordinate workers are required to maintain colonial homeostasis⁹, and therefore an equilibrium between subordinate and dominant types can be established.

The fitness differences between the subfamilies, however, do not necessarily require between-family competition. Direct individual fitness advantages of single workers are sufficient to explain the observed phenomena. Because only a few workers within a subfamily develop to pseudoqueens, there is also strong selection within the subfamily for reproductive dominance. Of course we cannot exclude nepotistic behaviour of the dominant patriline from our data, but it is certainly no requirement for the observed

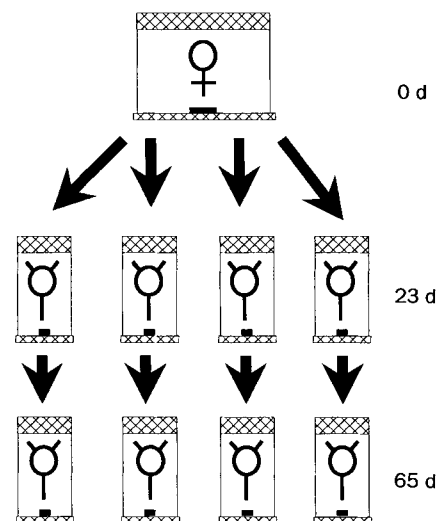


FIG. 1 Two colonies of *A. m. capensis* were dequeened and split into four equal-sized nuclei of about 4,000 bees each. Worker samples (about 40 per colony) were taken from the original colonies to determine the subfamily composition of the mother colony. After 23 and 65 days, worker brood samples (produced by laying workers) were taken from the splits. Furthermore, all queen larvae that were reared by the laying worker splits were collected. The samples were kept in ethanol until DNA processing, and were genotyped using single locus microsatellite fingerprinting as previously described^{5,6} (loci A14, A29, A76 and A107).

results, which fit well in a plain individual darwinistic fitness model.

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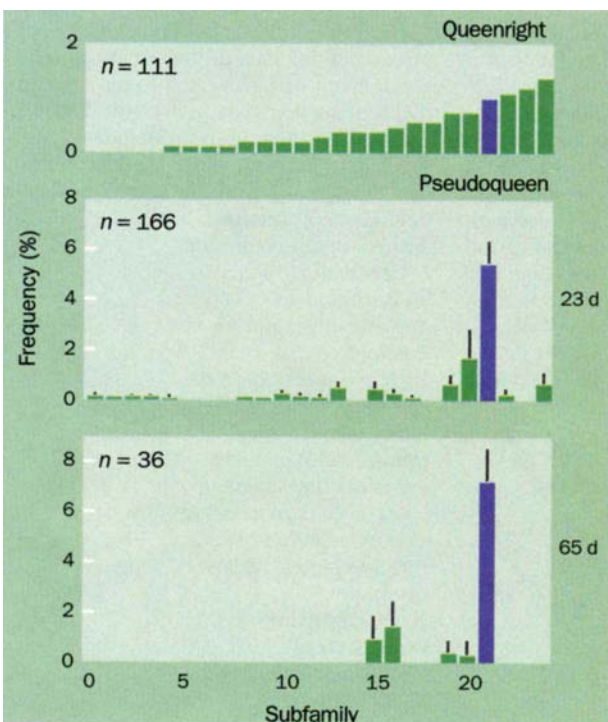


FIG. 2 Mean offspring frequencies (+ s.e.m.) of the various patrilineal observed in four split units of colony A (colony B not shown) over the observational period. The number of subfamilies in the original colonies A and B were 24 and 44, respectively, indicating a very high degree of polyandry in *Apis mellifera capensis*. This figure reduced dramatically after loss of the queen. Only five subfamilies in colonies A and B were present in the brood at the end of the observation period. One subfamily in each colony was clearly dominant (purple bars) and produced 8 out of 13 reared queens. The other five queens all belonged to different patrilineal.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and ten references.

Effect of natural tetrafluoromethane

SIR—Tetrafluoromethane (CF_4), a potent greenhouse gas¹ with an atmospheric lifetime of at least 50,000 years (ref. 2), is believed to be of purely anthropogenic origin. Here we present evidence that about half the current atmospheric burden of CF_4 has accumulated naturally from weak, probably radiogenic, sources in the lithosphere. We detected significant traces of CF_4 in natural gas, and extracted appreciable amounts of CF_4 and SF_6 from a fluorspar (CaF_2), containing traces of uranium. Both gases could in future be used as geochemical tracers to yield information on fluid transport in the Earth's crust and on the evolution of natural gas deposits. Additionally, determination of the abundance of CF_4 and SF_6 in cometary or other planetary atmospheres could help to characterize the prevailing radiochemical conditions.

Earlier stratospheric measurements of vertical concentration gradients of CF_4 and C_2F_6 indicated that CF_4 could be a natural constituent of the Earth's atmosphere³. To test this suggestion, other measurements had to be carried out. The Paul Scherrer Institute (PSI) kindly provided sections of an ice core from Colle Gnifetti (4,450 m above sea level) in the Monte Rosa massif at the Swiss/Italian border⁴. For dating of the core sections, we used ^{210}Pb measurements as well as stratigraphic signals (for example, debris from volcanic eruptions), corrected for the 40 years needed to achieve gas closure (T. Blunier, personal communication). We carried out gas analyses at the Max-Planck-Institut für Aeronomie (MPAE) based on the existing gas chromatography/mass-spectrometer (GC/MS) system with prior cryogenic pre-concentration⁵. After melting about 1 kg ice in a pressurized glass cylinder with a stainless steel

top, we extracted dissolved gases using two different approaches: 'bubbling out' with synthetic air for core section BC144 (gas closure in 1790 ± 30); and 'salting out' with sodium chloride for two sections of BC123 (gas closure in 1860 ± 15). We obtained good agreement within the precision ($\pm 20\%$) of both methods. We determined the volume of air extracted from closed pores of the core sections using simultaneous GC/MS-measurements of ^{126}Xe . We found a mean value of 39 ± 6 parts per trillion by volume (p.p.t.v.) of CF_4 for the pre-industrial atmosphere.

In addition to these ice-core measurements, we analysed air from two sealed glass vessels at MPAE. One (sample AU/Gö) was a 3-litre glass bottle from the 1950s, the other (BM60) a 30-cm³ glass sphere from December 1960. We opened both vessels in a water bath to avoid contamination by ambient air, and found that they possessed interior pressures of less than 500 hPa, showing that both vessels remained airtight after their production from the hot glass melt. The CF_4 mixing ratios did not show a significant deviation from the ice-core measurements.

The figure shows the reconstructed chronology of CF_4 based on our stratospheric measurements⁵ and the results of this work. It also includes the two emission models A and B for which historic production numbers of primary aluminium are multiplied with emission factors for CF_4 (1.1 and 2.3 kg t^{-1} , respectively). Scheme A assumes our measured background concentration, whereas scheme B starts with an atmosphere free of CF_4 . A shows good agreement with all our measurements and requires a realistic emission factor, whereas B reproduces our observations very poorly.

Because it is extremely unlikely that the pre-industrial background of CF_4 was

produced by early activities of humans, we had to identify natural sources of CF_4 for a conclusive picture. When trying to measure the efficiency of thermal destruction² of CF_4 in flames of natural gas, we found excessive CF_4 in the exhausts. The cryogenic pre-concentration technique is not suitable for the measurement of CF_4 directly in unburnt natural gas. But because flames of ultra-pure synthetic methane in ambient air did produce excessive CF_4 , we are confident that surplus CF_4 in natural gas flames is not produced through recombination of thermally dissociated fluorocarbons, but is indeed present with about 700 p.p.t.v. in the burnt Russian natural gas. If this value is representative for the whole world, no more than 3 t CF_4 would be emitted annually by the combustion of natural gas. This source would thus be only a minor contributor to the annual atmospheric input of 15,000 t CF_4 through other anthropogenic sources such as aluminium production⁵.

Previous mass spectrometric measurements⁶ provided evidence that CF_4 can be produced in fluoride minerals by radiochemical processes. With GC/MS we analysed extracted gases from the same types of fluorspar (Wölsendorf) and could unambiguously identify CF_4 as well as SF_6 with abundances of several parts per million, whereas C_2F_6 was undetectable. This radiogenic production mechanism of CF_4 seems to be responsible for the existence of natural atmospheric CF_4 through cold degassing from the Earth's crust, with an annual rate of between 0.1 and 10 t yr^{-1} , which is negligible compared with the current anthropogenic emissions.

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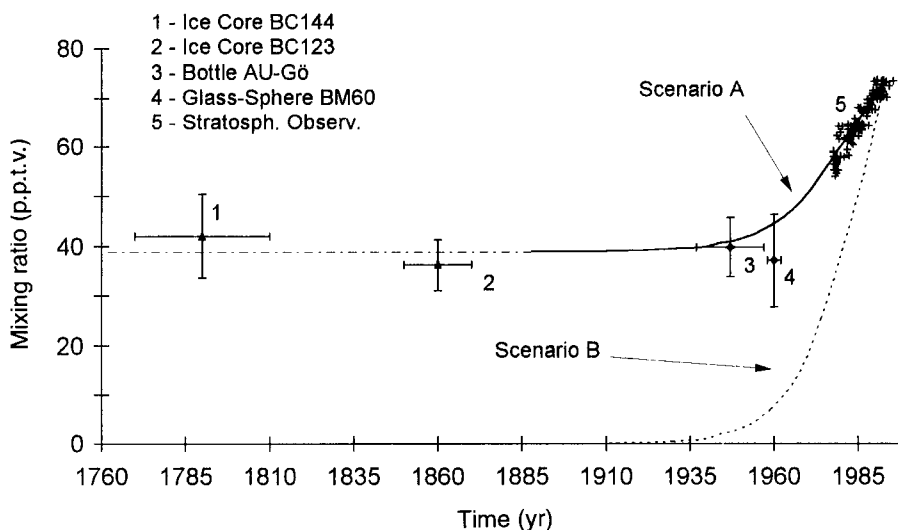
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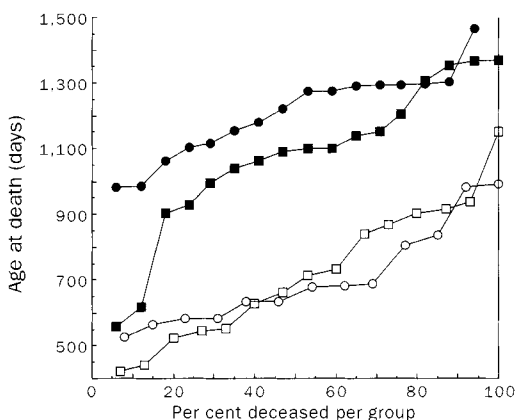
Reconstructed atmospheric chronology of tetrafluoromethane, with two emission models.

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Dwarf mice and the ageing process

SIR — Factors affecting longevity are complex and poorly understood. We have found that Ames dwarf mice (*df/df*), which are small and deficient in growth hormone (GH), prolactin and thyroid-stimulating hormone (TSH), live significantly longer ($P < 0.0001$) than their normal siblings. Hereditary dwarfism in mice may provide a valuable model for studying the ageing process.

The lifespan of an individual depends on genetic and environmental influences.



Longevity in male and female normal and Ames dwarf mice. Each point on the graph represents an individual animal surviving to the specific age indicated versus the percentage of animals deceased per group. Dwarfs live longer than normal mice regardless of gender ($P < 0.0001$). Mean age at death (days) $\pm$ s.e.m: normal male (open squares), 723 ± 54 ; normal female (open circles), 718 ± 45 ; dwarf male (closed squares), $1,076 \pm 56$; dwarf female (closed circles), $1,206 \pm 32$. One dwarf female is still alive.

The only well-documented method of extending lifespan is 'caloric restriction', as found in rodents. Here we report that genetically dwarf mice live much longer than normal animals from the same strain when maintained under standard laboratory conditions.

Ames dwarfs are characterized by primary pituitary deficiency consisting of the absence of, or extreme reduction in, anterior pituitary cells which produce and secrete GH, prolactin and TSH^{1,2}. They are of normal body size at birth but post-natal growth is severely retarded and the

body size of adult animals is approximately one-third of normal.

We maintained normal and dwarf mice in a conventional environment (not 'barrier' or specific-pathogen-free), fed lab chow and tap water without restriction. We checked daily for survival and general health 28 normal and 34 dwarf mice that were born during July and August 1992. Dwarf mice lived much longer than normal mice, with the difference in average lifespan being more than 350 days for males and more than 470 days for females ($P < 0.0001$). Two dwarf females reached the remarkable age of four years. These findings are particularly striking because Ames dwarfs exhibit some characteristics of reduced immune function³. Snell dwarf mice, which are deficient in the same three hormones as Ames dwarfs owing to a mutation on another chromosome, live longer than normal mice (K. Flurkey and D. E. Harrison, personal communication), although data have been reported that contradict this conclusion⁴.

Extension of lifespan in Ames and possibly Snell dwarf mice suggests that this effect is due to phenotype characteristics common to the two mutants: reduced body size and underlying endocrine defects. Small breeds of dogs and horses tend to live longer than larger breeds, and shorter people may live longer than taller people from the same population. Levels of insulin-like growth factor-I (IGF-I, mediator of GH action on growth) are lower in smaller breeds of dog⁵ and extremely low in Ames dwarfs⁶.

We suspect that GH/IGF-I deficiency is particularly relevant because overexpression of GH in transgenic mice is associated with markedly reduced lifespan and various indices of premature ageing⁷. Reduced lifespan has also been reported in patients with acromegaly and pituitary gigantism⁸. Dietary restriction reduces GH secretion and may be partially responsible for anti-ageing effects⁹.

Possible mechanisms of GH action on ageing include effects on metabolism, allocation of energy resources and sexual maturation. Deficiency of TSH resulting in hypothyroidism of Ames dwarfs could influence lifespan by reducing metabolic rate. However, in different species of homiothermic animals, slow metabolism and long lifespan coexist with large rather than small body size. Hypogonadism in Ames dwarfs could perhaps also contribute to their prolonged survival¹⁰.

We believe that genetically dwarf mice

are a valuable model for studying the mechanisms responsible for the ageing process and for setting the species-specific limits of survival.

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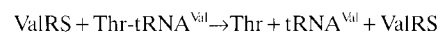
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Aminoacylation error correction

SIR — The amino-acid/trinucleotide relationships of the universal genetic code are established in aminoacylation reactions catalysed by transfer RNA (tRNA) synthetase enzymes¹. However, the fine discrimination of closely similar amino acids by binding interactions with synthetases is insufficient to account for the accuracy of the code². As a result, errors of aminoacylation occur³ which are corrected by specialized editing activities⁴⁻⁶ that have been conserved from bacteria to humans. Although crystal structures of many tRNA synthetases are now known, the basis for their editing activities has remained elusive. These editing activities include the ability to deacylate a mischarged tRNA. Two prominent enzymes with the deacylase activity are valyl- and isoleucyl-tRNA synthetases which deacylate Thr-tRNA^{Val} and Val-tRNA^{Ile}, respectively⁴⁻⁶. Each of these enzymes contains a novel insertion that splits the active-site-containing nucleotide binding fold. We present data here to show that the insertion cloned from valyl-tRNA synthetase (ValRS) deacylates Thr-tRNA^{Val} and that the one from isoleucyl-tRNA synthetase (IleRS) deacylates Val-tRNA^{Ile}.

Threonine, being isosteric with valine, is activated by ValRS at a frequency of about 1/350 to 1/400 (ref. 7). Upon addition of tRNA^{Val}, the misactivated threonine is hydrolysed both before and after the transfer of aminoacyl moiety to the 3' end of tRNA⁵. The 'post-transfer' editing reaction is:



In the isoleucine system, valine is activated by IleRS at 1/180 the rate of activation of isoleucine⁸. In the presence of

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tRNA^{Ile}, the enzyme also uses both pre- and post-transfer editing effectively to hydrolyse the misactivated valine^{3,6}.

ValRS and IleRS are examples of class I tRNA synthetases⁹, which contain a nucleotide binding fold made up of alternating β -strands and α -helices in an overall $\beta_6\alpha_4$ structure (Rossmann fold). The fold is divided into two $\beta_3\alpha_2$ halves by the connective polypeptide 1 (CP1)^{10,11} that is idiosyncratic to the protein. The structure of neither ValRS nor IleRS has been reported, but mutational analysis of each of these enzymes provides evidence that the sites for editing are distinct from those for amino-acid activation^{7,8}. Nevertheless, mutations in a defined segment of the CP1 region of IleRS alter the hydrolytic discrimination of mischarged Val-tRNA^{Val} versus correctly charged Ile-tRNA^{Ile} by IleRS¹². For this reason, we investigated further the role of CP1 in editing and, in particular, the possibility that this segment in isolation could deacylate a mischarged tRNA.

Figure 1 outlines the cloning of the 211-amino-acid CP1 segment of the 880-amino-acid *Bacillus stearothermophilus* ValRS (CP1_{Val}). Circular dichroism measurements and guanidine hydrochloride denaturation show that this fragment is a stable folded protein with some β -sheet structure (data not shown). We also successfully cloned two additional CP1 insertions: the 275-amino-acid CP1_{Ile} from the well-characterized 939-amino-acid *Escherichia coli* IleRS, and the 106-amino-acid CP1_{Met} from the 676-amino-acid *E. coli* methionyl-tRNA synthetase (MetRS). The last insertion was chosen as a control because MetRS does not deacylate mischarged tRNA^{Met} (ref. 13).

We first confirmed that ValRS selectively catalyses deacylation of mischarged Thr-tRNA^{Val} and not Val-tRNA^{Val}, and that IleRS specifically deacylates Val-tRNA^{Ile} (Fig. 2a). We then tested CP1_{Val} alone and found that, when added

to Thr-tRNA^{Val} and Val-tRNA^{Val}, the deacylation of Thr-tRNA^{Val} seen with ValRS was reproduced with the cloned insertion (Fig. 2b). Higher concentrations of CP1_{Val} (μ M) than of ValRS (nM) were required to observe the deacylase activity and this requirement seems to reflect the weak binding of the mischarged tRNA substrate to the cloned insertion. Because of the weak interaction, we were unable to determine an accurate k_{cat} or K_m for the reaction. However, we were able to assay for multiple substrate turnover by CP1_{Val}, indicating that the CP1 fragment is able to bind, catalyse and release the product (data not shown).

We observed little or no deacylation of Val-tRNA^{Val} by CP1_{Val}, even at the higher concentrations, showing that CP1_{Val} was specific for Thr-tRNA^{Val}. In a control experiment, we found that, in contrast to CP1_{Val}, CP1_{Met} has no activity directed towards Thr-tRNA^{Val} (Fig. 2b). This further confirms the high specificity of the CP1_{Val}-catalysed deacylation reaction. In view of these results, we tested whether CP1_{Ile} by analogy could catalyse deacylation of Val-tRNA^{Ile}. This cloned insertion selectively deacylated Val-tRNA^{Ile} and had little or no activity directed toward Ile-tRNA^{Ile} (Fig. 2c). This activity is specific to CP1_{Ile}, because addition of CP1_{Val} to Val-tRNA^{Ile} does not stimulate hydrolysis of the mischarged tRNA substrate. Thus, like CP1_{Val}, CP1_{Ile} has a deacylase activity with the same specificity as that of the enzyme from which it was cloned.

Among the 10 class I tRNA synthetases, ValRS and IleRS are part of a five-member subgroup of more closely related synthetases that also includes leucyl- (LeuRS), methionyl- and cysteinyl-tRNA synthetase (CysRS)¹¹. The CP1 insertions of MetRS and CysRS are relatively small (100 and 50 amino acids, respectively). Neither enzyme is known to deacylate mischarged tRNA. The other three enzymes all have large CP1 insertions ranging in *E. coli* from about

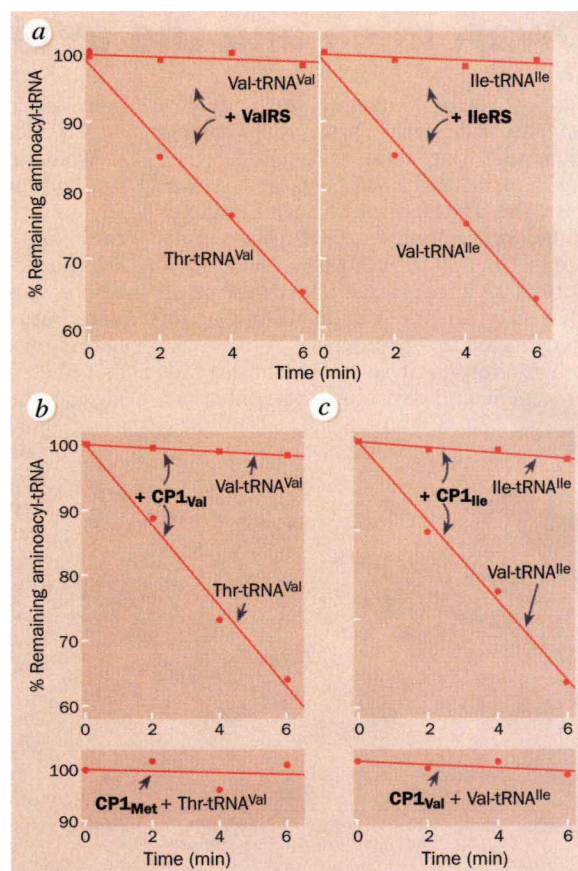


FIG. 2 Deacylation of mischarged tRNAs by ValRS and IleRS (a), or by CP1 fragments (b and c). Enzyme and substrate used in each assay were as labelled. The concentration for ValRS and IleRS was 2 nM, and that for CP1 fragments was 15 μ M. The aminoacyl-tRNA concentrations were 4 μ M in all cases. The background aminoacyl-tRNA hydrolysis in the absence of enzyme (less than 10% over 20 min) has been subtracted from these plots for normalization purposes.

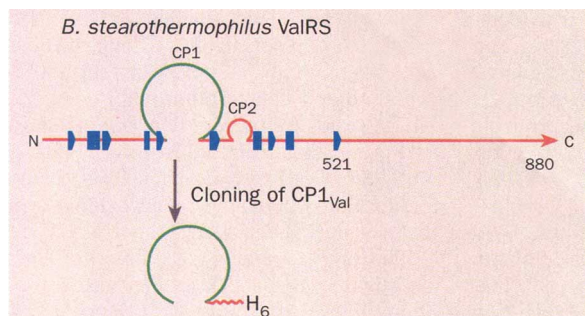


FIG. 1 Cloning of CP1 insertion of *B. stearothermophilus* ValRS. The isolated fragment is shown in red in the predicted secondary structure for ValRS. Blue arrows and rectangles designate β -sheets and α -helices, respectively. The coding sequence for CP1_{Val} (Leu 171 to Ala 372) was generated by introducing appropriate restriction sites into a phagemid harbouring the *B. stearothermophilus* ValS gene⁷. The CP1_{Val} gene fragment was then subcloned into an expression vector which provided an ATG start codon and a sequence encoding a 6xHis affinity tag at the C-terminal end of the recombinant protein.

250 to 275 amino acids. Like IleRS and ValRS, LeuRS catalyses deacylation of mischarged tRNA^{Leu} (ref. 14). We propose that a similar insertion in LeuRS also acts to correct errors of aminoacylation.

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Looking back in anger

Adrienne L. Zihlman

Demonic Males: Apes and the Origins of Human Violence. By Richard Wrangham and Dale Peterson. Houghton Mifflin: 1996. Pp. 332. \$22.95.

HUMAN violence is a topic of prime concern in most cultures, an expression of the dark side of human nature, a focus of research and a favourite ingredient in the daily headlines. The primatologist Richard Wrangham has joined the writer Dale Peterson to explore whether men's violent behaviour is cultural or has ancient roots in our ape ancestry.

In tracing the roots of human violence, the authors draw parallels between the human species and free-ranging chimpanzees at Gombe, Tanzania. Males from one group seemed to kill systematically and brutally former compatriots; survivors and their descendents now form a neighbouring community. This observation in 1974, the first of its kind after 13 years of chimpanzee watching, is often considered to reveal the ancestral origin of warfare and is the foundation for the authors' case that a unique combination of social characteristics is shared between humans and chimpanzees: that is, male-bonded communities and male-driven lethal intergroup raiding.

What develops early on as a considerable flaw in this argument is that the authors discuss in detail the behaviour of only one of the two species of chimpanzees. The genus *Pan* includes two species, *P. troglodytes* and *P. paniscus*, but, throughout the book, the authors use 'chimpanzee' to mean only *P. troglodytes*. The term 'bonobo' rather than the original term 'pygmy chimpanzee' is used for *P. paniscus*. This usage reflects valid disagreement within primatology but is sure to confuse and mislead a nonprimatologist. Calling *P. paniscus* bonobos wrongly implies that they are not chimpanzees, which they are. And, in their social behaviour, *P. paniscus* in many ways is the opposite to *P. troglodytes*.

Cross-cultural studies provide the authors with examples of the universality of male violence against men, against women and against members of other communities. The Yanomamo people from Brazil are well known for their lethal raids and counter-raids, and the raiders are allegedly the most genetically successful. There are no known human cultures, the authors report, that are rape-free or that have equality between the sexes. The island paradises of Paul Gauguin and Herman Melville existed only in their imaginations, and Margaret Mead's vision of Samoa was hopelessly astigmatic. Patriarchal male-bonded cultures are "world wide and his-

tory wide", a feature that "has its ultimate sources in male violence". Like chimpanzee males, men try to dominate their peers and want to win no matter what the cost.

Much of the book is devoted to dealing with violence in humans and apes — but not in *P. paniscus*. Males are found guilty of acting abominably towards members of the same species, hence the judgemental title of the book. Males form coalitions to raid and murder their neighbours; males rape and batter females and kill their infants. Yet females are obliged to turn to these very persecutors for protection. Females — ape and human — perpetuate this pattern through sexual selection by being attracted to the more aggressive males and bearing their progeny.

It is understandable why *P. paniscus* appears very late in the book and is dismissed as a deviant late arrival on the evolutionary scene, a dead end. Focusing on a *P. paniscus* 'model' rather than *P. troglodytes* would of course undermine the whole 'demonic' argument. In *P. paniscus* there is little aggression between males; males tend to avoid each other and stay close to their mothers. Males do not domi-

nate females, and relationships between males and females are congenial and highly sexual; encounters between communities are generally friendly.

The authors maintain that *P. paniscus* is highly specialized anatomically, as shown by their smaller shoulder blade. The authors emphasize similarities in anatomy and behaviour between *P. troglodytes* and gorillas. But genetically the two species are much closer to each other than to any other primate, and both species are equally distant from *Homo sapiens*.

Language here is a problem. Rather than describe as neutrally as possible what animals do and avoid tendentious interpretations that have specific meanings for human institutions or legal systems, the authors, from the title onward, schuss down the slushy slope describing ape behaviour in terms of assassination, murder, infanticide, battering and rape. After this downhill run, it seems easy to coast to the conclusion that the behaviours so described have the same genetic, developmental and social context for both apes and humans.

From the authors' point of view, *P. paniscus* is "a tale of vanquished demonism". They speculate that the species moved away from a violent ancestry because of the particular conditions of the environment. *P. paniscus* does not share its rainforest habitat with gorillas, the argument goes, allowing it to take advantage of the abundant herbs that are normally eaten by gorillas. The more abundant food



THE great apes have a diverse range of social organization, from the solitary lives of orang-utans to patriarchy in gorillas. *Great Ape Societies* describes recent research on all four species. The book, edited by William C. McGrew, Linda F. Marchant and Toshisada Nishida, opens with a foreword by Jane Goodall, famous for her fieldwork with chimpanzees, making a plea for conservation. Cambridge University Press, £55, \$64.95 (hbk), £19.95, \$24.95 (pbk).

source allows it to live in larger and more cohesive groups than can humans or *P. troglodytes*.

Furthermore, *P. paniscus* has not yet been observed to hunt monkeys, a behaviour found in *P. troglodytes*. Could it be, the authors ask, that the suppression of personal violence also suppresses predatory aggression? They conclude that "murder and hunting may be more closely tied together than we are used to thinking". In recalling Raymond Dart's emphasis on the predatory habit as "the mark of Cain" separating man from ape, the authors bring us full circle to recurring themes: connecting hunting and eating meat with aggression and war, and vegetarianism and eating herbs with a placid and peaceful nature. The theme was sounded in the story of Cain and Abel and in Robert Ardrey's best-selling *African Genesis* (1961), which introduced a generation of readers to meat-eating 'killer apes' — *Australopithecus africanus* preying on their herbivorous contemporaries, *A. robustus*. Before that we had H. G. Wells's *Time Machine*, in which demonic Morlocks of the distant future dine on the dumb but happy flower-eating Eloi.

In evolutionary science, there are no demons or angels. The capacity for aggression and violence, like the capacity for friendship and peaceful coexistence, exists in most individuals and human cultures, as it does in the apes and other mammals. The authors argue that both species of chimpanzee and humans descended from a violent common ancestor, but it could as easily be argued that both species of chimpanzee descended from a peaceable ancestor such as *P. paniscus*. In either case, our two closest living relatives together express a wider range of behaviours than either taken alone and offer a broader platform for speculating about the early history of our own multiplex genus and species. □

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Also on the dark side

Why is human violence so prevalent in the United States, especially among young single men? In *Violent Land: Single Men and Social Disorder from the Frontier to the Inner City* (Harvard University Press, \$29.95, £19.95), David Courtwright takes the long view of his subject, examining the interplay of biological, social and historical forces. Richard E. Nisbett and Dov Cohen, in *Culture of Honor: The Psychology of Violence in the South* (Westview, \$59.95, £44.50 (hbk), \$12.95, £8.95 (pbk)), conclude that the higher rate of homicide among whites in the southern United States is due to the traditional 'culture of honour', in which a man's reputation is seen as central to his economic survival.

Myths about a polymath

Bernadette Bensaude-Vincent

Arrhenius: From Ionic Theory to the Greenhouse Effect. By Elisabeth Crawford. *Science History Publications*: 1996. Pp. 320. \$49.95.

To many students of chemistry, Arrhenius is just a name attached to an equation. Elisabeth Crawford's biography of the Swedish scientist unveils the face behind the name, and reveals a human being living, loving and fighting against his colleagues. Svante August Arrhenius (1859–1927) is recognized as one of the founders of physical chemistry, but until now there has been no complete biography, and all the published sketches repeat standard accounts of his work formulated by Arrhenius and his brother-in-law.



Arrhenius: expert at promoting myths about himself.

Crawford enjoys a high reputation for her historical studies of the Nobel Institute and of international relations in science. Her familiarity with the Swedish language and culture allowed her to research this new view of the life of Arrhenius the scientist. Crawford provides a fine analysis of Arrhenius's work on electrolytic dissociation and immunochemistry, based on archival material in the Arrhenius Collection at the Royal Swedish Academy. She reconstructs his investigative pathway in some detail, making the technical aspects accessible to lay readers through careful definitions of the terms.

The mythical image of Arrhenius's early career, which he built up after becoming famous for winning the Nobel prize for chemistry in 1903, is subjected to careful scrutiny. In speeches, Arrhenius portrayed himself as a young creative discoverer who became the victim of narrow-minded and conservative colleagues. This was motivated by a key event in his life, the humiliation he felt because he did not receive the highest grade for his doctoral dissertation

— the usual opening to an academic career. (He was awarded only a fourth class, the lowest possible pass.) He then became resentful towards the Uppsala physicists who condemned all attempts at theoretical interpretations.

But in fact Arrhenius's 1884 doctoral dissertation, aimed at determining molecular weights by conductivity measurements, did not contain his theory of electrolytic dissociation and his famous equation, which were established only in 1887. One of the most valuable chapters in the book examines the influence exerted on the development of Arrhenius's theory by Jacobus van't Hoff's memoirs on the law of chemical equilibrium, published in 1886. A popular myth is that the theory of the 'ionists' first met hostility in the scientific community and generated great controversy. Crawford counteracts this with evidence of a contrast between a handful of opponents and the great mobilization of Friedrich Wilhelm Ostwald and the "army of ionists" who, through an active campaign, worked

for a wide circulation and rapid acceptance of the notion of permanent ions.

The most original feature of the book is its emphasis on local context and its influence on Arrhenius's scientific style. As a postdoctoral fellow, he travelled in Germany and Austria where he was able to collaborate with Ostwald, Walter Nernst and Ludwig Boltzmann. Although Arrhenius gained an international reputation, he decided to make a career in his native Sweden, which was then on the periphery of the major scientific powers. He succeeded Anders Jonas

Ångström at the Royal Institute of Technology in Stockholm where teaching duties were light and laboratory facilities were not up to the usual standards of an academic institution.

In this niche, Arrhenius defined a style of his own. Like Louis Pasteur, he continually changed his field of research. After his trail-blazing contribution to the beginnings of physical chemistry, he moved to cosmic physics where he became interested in cyclical phenomena. He predicted the influence of carbon dioxide in the atmosphere on climate changes — later known as the greenhouse effect — and ventured that this could explain the ice ages. In the 1890s, he tried to apply physical chemistry to the study of the reactions between toxins and antitoxins, became involved in a long controversy with Paul Ehrlich over Ehrlich's theory that certain antibodies had side chains that could bind specific toxin molecules, and eventually positioned himself as the founder of 'immunochemistry'.

Arrhenius became acquainted with new fields of research through informal com-

munication — conversations with new friends or travels to learn experimental skills in laboratories specialized in the area he wanted to investigate. In this way he became a kind of specialist in the hybridization of academic disciplines. In Stockholm, his work on cosmic physics was developed through interaction with a meteorologist, a geologist, an astronomer and a physico-chemist. Arrhenius never wanted to secure an institutional basis for his informal research group. He did the same when he entered biochemistry through his collaboration with a Danish scientist, Madsen.

Instead of spending time developing institutions, he devoted his energy to controversies — with Nernst and Ehrlich for instance — and to popular lectures and writing that extended his fame far beyond a circle of academic scientists. Crawford

argues that there was a public audience for science in Sweden when the Nobel prizes were founded. This reinforced Arrhenius's reputation and provided him with a well-equipped Nobel Institute for Physical Chemistry. Despite the marginal and nonacademic style he had chosen, Arrhenius became a central figure and a powerful international academic authority.

Crawford not only reveals the diversity of Arrhenius's contributions to science but also opens a window on the international scientific community of a century ago. Her book does more than fill a biographical gap. □

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To sleep, perchance to learn

Mary A. Carskadon

The Enchanted World of Sleep. By Peretz Lavie. Yale University Press: 1996. Pp. 270. \$27.50, £18.95.

PERETZ Lavie weaves a magical spell about sleep science through an often personal yet scientifically accurate vision in this very readable small book. Originally written in Hebrew and received with popular acclaim in Israel, the English translation retains its charm for the lay reader and interested scientist alike.

Two principal features contribute to the reader's enjoyment. The first is Lavie's penchant for providing historical context and human-interest insights about the investigators who have been instrumental in unmasking sleep's mysteries. The second feature is afforded by the broad landscape of Lavie's own research experience, which allows him to explain difficult concepts with data conjured up in his own laboratory.

The foreword by Michel Jouvet begins the enchantment with a primordial view of life's rhythms. Lavie then whisks forward to the present-day sleep laboratory for an entrancing tale of sleep and dreams. He traces Greek and Judaic traditions and quickly moves from the theories of Alcmaeon to Pavlovian conditioning. Modern approaches to studying the sleeping brain are described with clarity, from the primitive electroencephalograph recordings of Caton and Beck to the tale of Hans Berger, of whose tragic demise we also learn. (Just when the reader begins to wonder how anyone can possibly sleep while all wired up in the laboratory, Lavie reveals that more than 15,000 people have been studied in the Technion Sleep Laboratory — and those who have failed to fall asleep can be num-

bered on the fingers of one's hands.)

Lavie is at his best when he profiles sleep researchers. We learn, for example, that Nathaniel Kleitman, born in Russia, found his way to the University of Chicago by way of Beirut, Rhodes, Columbia University and the University of Georgia. Kleitman's passionate investment in the study of sleep, if not academic suicide, certainly was not regarded by him as the academic fast track. The account of the seminal years at Kleitman's Chicago laboratory gives readers a front-row view of the discovery of rapid eye movement (REM) sleep.

The normal rhythms of sleep and wakefulness, the ebb and flow of REM and non-REM sleep, are given great salience by Lavie's descriptions of "sleep gates" and "forbidden zones" for sleep of the pineal gland, and of the "darkness hormone", melatonin. Lavie also describes his own theory that REM sleep functions to provide a "gateway to wakefulness" during the night, the glue that permits sleep to consolidate. We learn, too, of another REM theory, proposed by Francis Crick. It is delicious to read Lavie's description of his own patient — an Israeli defence-force veteran with a shrapnel wound to his brain stem who had no REM sleep — and to listen in on Lavie's conversations with Crick about the implications of this patient's condition for Crick's theory.

Lavie introduces the main sleep disorders, always giving a central example to aid understanding. Situational insomnia, for example, is made more meaningful by reading of the sleep problems many experienced during the Gulf War under the threat of Scud missile attacks. Then we learn of Lavie's creative population-wide cure for situational insomnia. And the psychological role that the persistence of a

Sweet dreams: the sleep laboratory unlocks the secrets of slumber.

sleep disorder plays for some patients is eloquently portrayed by citing Proust's aunt in *Remembrance of Things Past*. Lady Macbeth, Sancho Panza, Stephen Jay Gould, Constantin von Economo, Oliver Sacks, Holocaust victims, Robert Louis Stevenson, Lavie's mentors, colleagues and students, and many more populate these pages and enrich the work.

Lavie has produced a literate, informative and entertaining read for those who wish to delve into the mystic realms of sleep and dreams. □

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... and writing, reading, talking

The World on Paper: The Conceptual and Cognitive Implications of Writing and Reading by David R. Olson. Cambridge

University Press, £12.95 (pbk). Reviewing the hardback edition in these pages, John C. Marshall described the book as "fascinating (and infuriating).... [The author's] grandiose claim is that we 'read' both the book of nature and the book of humanity differently after the world has been put on paper". "On my reading of his text at least," Marshall concluded, "the counter-evidence far outweighs the corroboration". *Nature* **371**, 665 (1994).

Language, Learnability and Language

Development by Steven Pinker. Harvard University Press, \$18.95, £12.50 (pbk). A classic study presenting a comprehensive theory of child language acquisition. First published in 1984, it is now reprinted with a new commentary by the author that updates every section.

Building planetary surfaces

Lionel Wilson

Volcanoes of the Solar System. By Charles Frankel. Cambridge University Press: 1996. Pp. 232. \$69.95, £40 (hbk); \$24.95, £14 (pbk).

THE planetary exploration missions of the past two decades have shown that volcanic activity is by far the most important mechanism forming the crusts of the silicate-dominated bodies in the Solar System. Volcanic eruptions in various guises and at various times have constructed more than three-quarters of the surface features of Earth and Venus, half of the surface area of Mars and at least a quarter of the surface of our Moon — even more in both of the latter cases if one counts as a volcanic

kinetic energy of accretion itself, was the decay of short-lived radioactive isotopes, activity was inevitably confined to the early part of Solar System history. The larger bodies, with smaller ratios of surface area to volume, and so a greater efficiency of heat generation relative to heat loss, have been forced to cope with the energy produced by the long-lived isotopes of potassium, uranium and thorium, the main elements that power Earth and (almost certainly) Venus. Jupiter's satellite Io, although smaller than the Moon, is still vigorously volcanically active today, but only because it derives heat from the enormous body tide generated within it by nearby Jupiter as two of the other satellites, Europa and Ganymede, exert mutual gravitational nudges that distort its orbit — and lead to their own heating, albeit at much lower levels.

All the above issues are presented and discussed with disarming simplicity in Charles Frankel's excellent summary of planetary volcanism. High-quality illustrations give examples of the main types of volcanic feature (lava flows, ash deposits, volcanic mountains, and collapse and explosion craters) visible on all the active bodies in the Solar System. If this were all that the book accomplished, I would count it as a useful addition to the literature. But there is much more. Frankel describes in great detail the physical principles that control the formation of volcanic features, showing how the sizes and shapes of edifices and deposits are related to the amounts and physicochemical properties (viscosity, dissolved volatile content and so on) of magmas reaching the surfaces of planets and to the environmental

conditions into which they emerge. It is specifically this comparison of the sizes, shapes and chemical compositions of volcanic deposits formed under differing environmental conditions (especially those of gravity, atmospheric pressure and crustal stress) that has led to so many advances during the past 20 years in the understanding of volcanism as a general process.

Attempting to deal with so much basic geology and geophysics in a work aimed mainly at a general audience must have been a daunting task, but Frankel has succeeded amazingly well in assimilating and conveying to his readers the essentials of modern theoretical volcanology as applied to both Earth and the other planets. Of course, many issues are simplified, although I found nothing that I regard as a major error. Indeed, the simplification almost always manages to leave the reader with the correct general impression of

what is going on, so that only further elaboration, rather than a complete return to basics, would be needed to present a more sophisticated story. I was repeatedly delighted to see how well this had been done — an object lesson to all writers of first-year undergraduate textbooks.

Several highlights stand out: the engaging accounts of the Apollo astronauts doing their best to carry out field geology in the extremely harsh lunar environment, followed by an erudite discussion of what the rocks collected on the Apollo missions have told us about lunar geological history and, by implication and inference, the history of the larger planets; a description of a hypothetical field trip to the summit area of the Martian volcano Olympus Mons, setting the scene for an understanding of the immense lengths of time during which some Martian volcanoes were active over hot-spots in the mantle; and a reminder of the excitement (terror?) of standing near a flow of liquid sulphur on Io and watching its completely unexpected behaviour as, for a while, it becomes more fluid, rather than less fluid, as it cools.

As an up-to-date, easily readable, accurate and enjoyable introduction to all aspects of planetary volcanology, I cannot imagine this book being bettered for some time. □

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IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Volcanoes build planet surfaces: eruption on Io.

phenomenon the solidification of the global molten-magma oceans generated by the rapid accretion of these bodies. Only Mercury among the inner planets has still to be imaged at high enough resolution for specific volcanic features on its surface to be recognized unequivocally. And recent analyses of some of the rocks in the world's meteorite collections have shown that several small asteroids have had significant volcanic histories.

In retrospect, we should not be surprised that volcanism is so widespread: advective transport of molten rock to the surface of a planet is a much more efficient method of removing heat from the interior than thermal conduction through the essentially rigid crust. The timing and duration of volcanic activity on a body is a delicately balanced matter, however. With the smaller bodies, for which the most important heat source, other than the

Back on Earth (in paperback)

Principles of Precambrian Geology by Alan M. Goodwin. Academic, £35 (pbk). An abridgement of the author's acclaimed book *Precambrian Geology*, published in 1991.

Human Impact on the Earth by William B. Meyer. Cambridge University Press, £40, \$69.95 (hbk), £12.95, \$24.95 (pbk). A slimmed-down, updated version of the landmark volume *The Earth as Transformed by Human Action* edited by B. L. Turner *et al.*, which was described by Cynthia Rosenzweig and Daniel Hillel in these pages as "an indispensable reference and resource on global change... instructive and challenging". *Nature* **355**, 781 (1992).

Global Tectonics by Philip Kearey and Frederick J. Vine, second edition. Blackwell, £22.50 (pbk). A textbook intended for senior undergraduates in the geological science, and postgraduate students and other geoscientists who wish to gain an insight into the subject. In his review of the first edition, however, Peter J. Smith complained that "from the teaching point of view the book is not quite basic enough... it too often fails to explain". *Nature* **350**, 200 (1990).

The PPAR α –leukotriene B₄ pathway to inflammation control

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Inflammation is a local immune response to 'foreign' molecules, infection and injury. Leukotriene B₄, a potent chemotactic agent that initiates, coordinates, sustains and amplifies the inflammatory response, is shown to be an activating ligand for the transcription factor PPAR α . Because PPAR α regulates the oxidative degradation of fatty acids and their derivatives, like this lipid mediator, a feedback mechanism is proposed that controls the duration of an inflammatory response and the clearance of leukotriene B₄ in the liver. Thus PPAR α offers a new route to the development of anti- or pro-inflammatory reagents.

In many inflammatory disease states the turnover of the dihydroxy fatty acid leukotriene B₄ (LTB₄) determines the extent and duration of inflammation (for review, see ref. 1). LTB₄ induces a complex cascade of molecular and cellular events that ultimately recruit cells from the immune system to the site of injury and produce an inflammation. It is inactivated through metabolic degradation by the microsomal ω - and peroxisomal β -oxidation pathways^{2,3}.

Catabolism of LTB₄ occurs primarily in cells of the immune system at the site of inflammation⁴ and also in hepatocytes, liver being the principle organ for clearance of LTB₄ from the blood circulation⁵. Many compounds, for example, dietary polyunsaturated ω -3 fatty acids (ω -3 PUFAs⁶), upregulate the degradation of LTB₄ in both cell types, suggesting the existence of a common regulatory pathway. Clofibrate is a hypolipidaemic (lipid-lowering) drug and peroxisome proliferator which increases peroxisomal enzymes in cultured mouse macrophages⁷. Catabolism of LTB₄ is induced in primary hepatocytes from clofibrate-treated rats as a result of increased expression of genes encoding the peroxisomal β -oxidation enzymes⁸. These findings, considered with the proposed intracellular site(s) of action of LTB₄ (ref. 9) and its effect on gene expression¹⁰, indicate that LTB₄ inactivation may be modulated by transcriptional regulation of the inducible ω - and β -oxidation pathways, and that this process could be influenced by specific activators such as PUFAs, clofibrate and other peroxisome proliferators.

The peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors that regulate gene expression of enzymes associated with lipid homeostasis, including fatty acid degradation (see ref. 11 for a review). PPARs are members of the nuclear hormone-receptor family named by virtue of their ability to mediate a pleiotropic response to peroxisome proliferators (PPs)^{12,13}. These receptors can be activated by hypolipidaemic fibrates such as clofibrate, some arachidonic acid metabolites, and various fatty acids^{14–17}. So far, three subtypes have been identified (α , β/δ or FAAR and γ) from *Xenopus*¹⁸, rodents^{13,14,18–22} and humans^{23–25}. Transcriptional regulation by PPARs is achieved through PPAR–RXR (where RXR is the receptor for 9-*cis* retinoic acid) heterodimers which bind to DNA motifs termed PPAR-response elements (PPREs) in the promoters of their target genes^{16,26}. Analysis of PPAR α knockout (–/–) mice indi-

cates that the effects of PPs such as the hypolipidaemic agents Wy14,643 and clofibrate, are mediated by PPAR α ²⁷. PPAR α is primarily expressed in tissues that have a high fatty acid catabolism, including the liver and the immune system^{14,28}. In the liver, the functions of PPAR α are to degrade fatty acids and detoxify various xenobiotics, both of which are achieved by the ω - and β -oxidation pathways⁶. The role(s) of PPAR α in other tissues is not known.

Structural similarities of PPAR with the ligand-binding domain of other members of the nuclear hormone-receptor superfamily identify the carboxy-terminal domain of PPAR α as a putative ligand-binding domain¹¹. The prostaglandin 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (refs 29, 30) and the antidiabetic thiazolidinediones³¹ are both ligands for the PPAR γ subtype, but so far no PPAR α ligands, natural or synthetic, have been reported.

These findings reveal a connection between LTB₄ catabolism by microsomal ω - and peroxisomal β -oxidation, the inducers of this process, and the function, distribution and activation of PPAR α . We have therefore investigated whether PPAR α regulates LTB₄ inactivation and thus determines the duration of an inflammatory response and clearance of LTB₄ in the liver. We first show that LTB₄ is an activator and a bona fide natural ligand of PPAR α . Activation of PPAR α results in the induction of genes involved in the fatty acid oxidation pathways that degrade fatty acids and fatty acid derivatives like LTB₄. Finally, in agreement with these findings, we show *in vivo* that PPAR α affects the duration of an inflammatory response induced by LTB₄ or its metabolic precursor arachidonic acid.

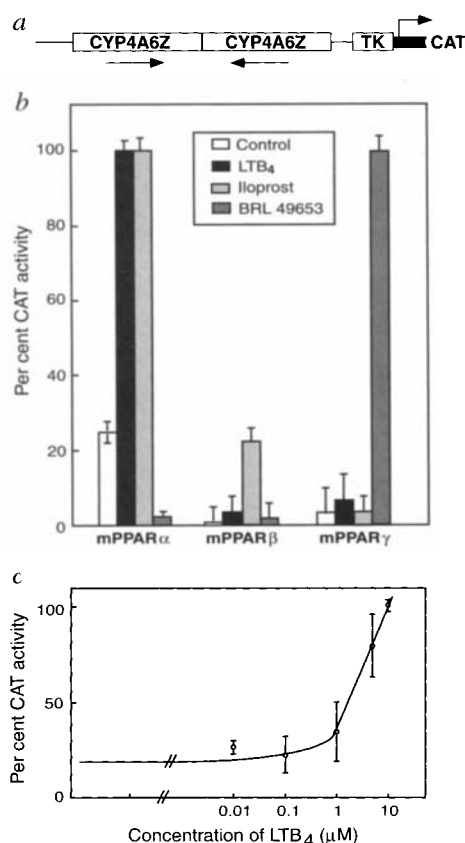
Detection of PPAR α activation

Standard transient cotransfection assays were used to measure PPAR activation response. Cells were cotransfected with an internal control standard, an expression plasmid for PPAR, and a reporter plasmid expressing the chloramphenicol acetyltransferase (CAT) gene under control of a PPRE. In the presence of an activator, PPAR binds as a heterodimer with RXR to the PPRE DNA response element and activates transcription of the CAT gene, resulting in increased CAT activity.

The sensitivity of this assay is dependent on cell type, receptor and DNA response element. We first optimized the sensitivity of our HeLa cell system by using a suitable reporter plasmid containing an optimal DNA response element. The activator Wy14,643, a peroxisome proliferator, was used to evaluate response of wild-type PPARs from reporter plasmid constructs containing PPREs from different target genes. The best response, in terms of

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FIG. 1 Activation of PPAR α by LTB $_4$ in transient transfection assays. *a*, Reporter plasmid for transfection assays. The construct contains the CAT coding sequence driven by a promoter consisting of two copies of the CYP4A6 PPRE (in a palindromic configuration) upstream of the thymidine kinase minimal promoter. *b*, Basal levels of CAT activity (white bars) and LTB $_4$ -induced CAT activity (filled bars). mPPAR α is specifically induced by 10 μ M LTB $_4$ whereas mPPAR β and γ show no significant response. For LTB $_4$ and iloprost, 100% CAT activity was arbitrarily chosen to correspond to the activation of PPAR α by 10 μ M activator; for BRL 49653, 100% activity corresponds to the observed activation of PPAR γ . *c*, Dose-response curve of mPPAR α . The concentration of LTB $_4$ that results in half-maximal PPAR α activation (EC_{50}) is in the micromolar range.



increased induction, was obtained with a reporter construct containing two copies of the CYP4A6 PPRE in a palindromic configuration (Fig. 1*a*, and our unpublished results).

LTB $_4$ specifically activates PPAR α

The transient transfection assays were used to test the ability of LTB $_4$ and its metabolites to activate PPARs. Figure 1*b* shows that LTB $_4$ specifically activates mouse PPAR α (mPPAR α) but not mPPAR β or γ . By contrast, the LTB $_4$ catabolites 20-OH and 20-COOH LTB $_4$ did not significantly activate the PPARs (data not shown). The dose-response curve for the activation of PPAR α by LTB $_4$ shows a typical dose-dependent pattern in which the response (in CAT activity) increases with increasing amounts of activator. In our HeLa cell assay system, the effective concentration of LTB $_4$ in the culture medium required for half-maximal response (EC_{50}) is in the micromolar range (Fig. 1*c*). This EC_{50} value is comparable to those observed in the induction of PPAR γ by its ligand 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ (refs 29, 30). A relatively high EC_{50} was expected as LTB $_4$ is a chemically and metabolically unstable compound and the efficiency of its uptake and catabolism is dependent on cell type³². Similar activation results were obtained with PPAR α from *Xenopus* (xPPAR α) and humans (hPPAR α) (data not shown), showing that this response is conserved from lower vertebrates to humans.

We next verified that this PPAR-subtype specificity was due to selective activation of PPAR α by LTB $_4$ and not to differences in expression of PPAR α , β and γ . Bandshift assays of extracts from PPAR-transfected HeLa cells and a PPRE-containing oligonucleotide indicated that all three proteins were expressed and able to bind a PPRE. The receptor levels varied only slightly in the ratio of 2:3:1 for α : β : γ (data not shown), supporting a selective activation of PPAR α by LTB $_4$. The presence of functional PPAR β and γ subtypes in transfected HeLa cells was confirmed by their activation by other known PPAR activators (the antidiabetic thiazolidinedione BRL49653 and the anti-inflammatory agent iloprost; Fig. 1*a*).

LTB $_4$ upregulates PPAR α target genes

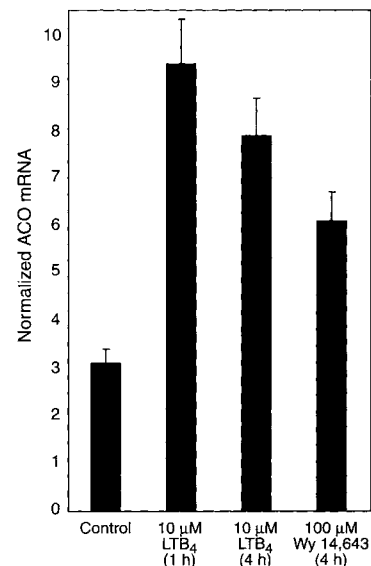
Our transient transfection assay identified LTB $_4$ as an activator of PPAR α , so the next step was to put this activation into a biological context. The liver is the primary organ for LTB $_4$ clearance from the blood circulation. In primary hepatocytes, LTB $_4$ catabolism is induced by the PPAR α activators clofibrate and PUFAs⁸. PPAR α activation can be assayed in primary hepatocyte cultures by monitoring the increase in messenger RNA encoding acyl Co-A oxidase (ACO), which correlates with an increase in ACO protein³³: as ACO is the rate-limiting enzyme in the peroxisomal β -oxidation pathway, an increase in ACO mRNA reflects induction of this pathway. We have used this assay to show that LTB $_4$ increases transcription from PPAR α -responsive genes. Primary hepatocyte cultures were incubated in the presence of various activators, RNA was isolated, and the ACO mRNA levels were quantified by RNase protection assay. Levels of ACO mRNA were normalized to those of the mRNA of ribosomal protein L27, whose expression is not regulated by PPARs. As the turnover of LTB $_4$ is normally high, the effects of ACO mRNA expression were evaluated in the first few hours after exposure to LTB $_4$. This treatment led to a three-fold increase in expression of ACO mRNA (Fig. 2). As a control, we used the potent PPAR α activator Wy14,643 to show that the induced expression of ACO mRNA was twofold after 4 hours (Fig. 2). These results indicate that LTB $_4$ increases transcription of a PPAR-regulated gene whose product is rate-limiting in the degradation pathway of fatty acids. The extent of induction by this natural lipid mediator is comparable to that observed with the potent synthetic hypolipidaemic compound Wy14,643.

LTB $_4$ and Wy14,643 are PPAR α ligands

Many compounds, including fibrates, fatty acids and eicosanoids, will activate PPAR α , but the mechanism of this activation is unknown. The selective activation by LTB $_4$ of PPAR α to induce transcription from target DNA response elements and increase transcription from PPAR target genes could be the result of direct interaction between PPAR α and LTB $_4$ or of an indirect LTB $_4$ -mediated pathway.

To clarify the nature of the PPAR α -activation mechanism, we tested whether LTB $_4$ was a PPAR α ligand in an assay that exploits the structural organization of PPARs into independent functional domains¹¹. We used a bacterially expressed fusion protein of glutathione-S-transferase (GST) with the ligand-binding domain of PPAR α (PPAR α (LBD)) to evaluate binding to radiolabelled

FIG. 2 In primary hepatocyte cultures, LTB $_4$ activates transcription of genes involved in the β -oxidation pathway. Transcripts from the acyl Co-A oxidase (ACO) gene are increased by exposure of hepatocytes to 10 μ M LTB $_4$. This effect can be detected as early as 1 h (three-fold) after treatment, and persists for at least 4 h (2.5-fold). The expected induction is observed when cells are treated with the hypolipidaemic drug Wy14,643 (twofold for 4 h).



LTB₄. The result was a typical saturation binding curve (Fig. 3a). Scatchard analysis (Fig. 3a, inset) gave a dissociation constant (K_d) of ~90 nM for the binding of LTB₄ to PPAR α . Unlabelled LTB₄ could compete with radiolabelled LTB₄ for PPAR α binding (Fig. 3b), whereas compounds that do not activate PPAR α , a monounsaturated fatty acid (erucic acid¹⁶; Fig. 3b) and 17 β -oestradiol (Fig. 4), could not. The hypolipidaemic drug Wy14,643 also bound specifically to PPAR α (Fig. 4), although less efficiently than LTB₄ (45% versus 80% competition; Fig. 4). These results indicate that activation of PPAR α by LTB₄ and Wy14,643 is due to binding of these compounds to PPAR α . The natural inflammation mediator LTB₄ and the synthetic hypolipidaemic drug Wy14,643 are both ligands for PPAR α .

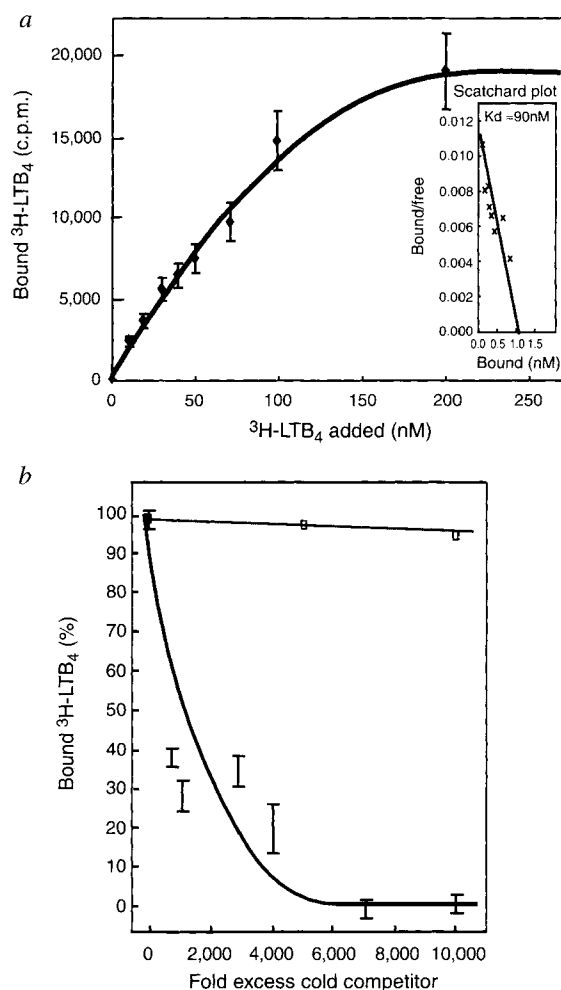


FIG. 3 LTB₄ is a ligand for PPAR α . The GST-PPAR α (LBD) fusion protein contains amino acids 171–474 of the wild-type xPPAR α protein. The results reflect three independent trials done in duplicate. a, Saturation binding curve from the specific interaction of PPAR α with LTB₄. Purified GST-PPAR α (LBD) fusion proteins were incubated to equilibrium with increasing amounts of ³H-LTB₄. Bound ³H-LTB₄ fraction was separated on a size-exclusion column and counted. Nonspecific binding of ³H-LTB₄ was determined as binding to the GST portion of the fusion protein. A dissociation constant (K_d) of ~90 nM was determined by Scatchard analysis (inset) (different experiments gave values between 50 and 130 nM). b, Competition binding. Per cent bound refers to the c.p.m. in the bound fraction as a percentage of the c.p.m. when no competitor is present. 50% specific competition for PPAR α requires ~1,000-fold excess of cold LTB₄. Erucic acid, a monounsaturated C₂₂ fatty acid that does not activate PPAR α ¹⁶, does not compete for binding. Contribution of nonspecific binding of ³H-LTB₄ was determined as binding in the presence of 10,000-fold excess unlabelled LTB₄.

PPAR α and the inflammatory response

At the site of inflammation, the levels and degradation rate of LTB₄ determine the extent and duration of an inflammatory response. Direct interaction between PPAR α and LTB₄ induces enzymes for fatty acid degradation and increases catabolism of LTB₄. Thus PPAR α is potentially a modulator of the duration of LTB₄-mediated inflammation, a role that can be tested by comparing the inflammatory response in PPAR α wild-type (+/+) and knockout (-/-) mice.

A standardized *in vivo* assay for inflammation is the mouse ear-swelling test (MEST)³⁴, in which the inflammatory agent is topically applied to the ear; ear thickness is monitored as a measure of inflammation. Standard assays utilize two compounds that trigger two different inflammatory pathways: phorbol esters (TPA (12-*O*-tetradecanoylphorbol-13-acetate)) and arachidonic acid³⁵. The phorbol ester model is characterized by prostaglandin components³⁶, whereas arachidonic acid can be metabolised to many different chemotactic lipid mediators, including the very potent leukotrienes³⁷. Inflammation induced in mice that cannot metabolize arachidonic acid to leukotrienes because of a disrupted 5-lipoxygenase gene, indicates that LTB₄ is a component of arachidonic acid-mediated inflammation³⁸.

The MEST assay was used to compare the inflammatory response in PPAR α (+/+) and (-/-) mice. Three inflammatory agents were tested: arachidonic acid, LTB₄ and the phorbol ester TPA. Inflammation due to either arachidonic acid (Fig. 5a) or its more potent derivative, LTB₄ (Fig. 5b), is prolonged in mice with a disrupted PPAR α gene as compared to wild-type mice. In support of our proposed link between LTB₄ clearance and PPAR α activity, the response to TPA (Fig. 5c) is similar in (+/+) and (-/-) mice. These results show that PPAR α affects the duration of inflammation induced by LTB₄/arachidonic acid: the lack of PPAR α activity extends the inflammatory response.

Our results also reveal that the β and γ PPAR subtypes do not compensate for a lack of PPAR α in an LTB₄-mediated inflammatory response. This is consistent with the HeLa cell transfection assays which indicated that LTB₄ specifically activates PPAR α and not PPAR β or γ .

Extension rather than persistence of the inflammatory response in our PPAR α knockout mice is consistent with their phenotype: PPAR α knockout mice are apparently healthy and develop and

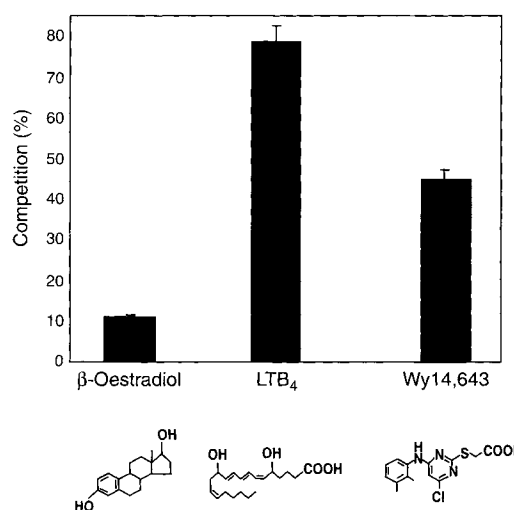


FIG. 4 The hypolipidaemic agent Wy14,643 is a ligand for PPAR α . Competition experiments for ³H-LTB₄. Wy14,643 and LTB₄ compete for PPAR α binding, indicating that they must be ligands. Competition with 17- β -oestradiol, a compound that does not activate PPAR α , acts as a control to demonstrate the specificity of the reaction. The per cent competition refers to the decrease in the amount of radiolabelled ligand bound to the protein compared to the amount when no competitor is present. Competitors were added at 10,000-fold excess.

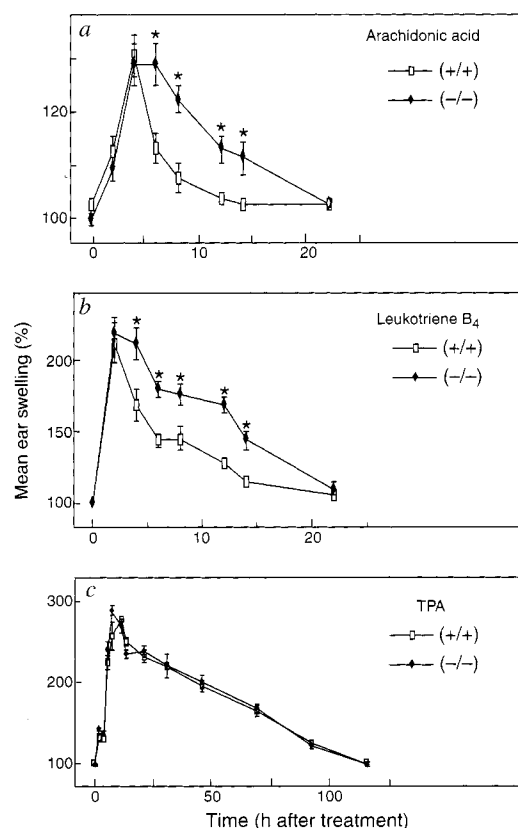


FIG. 5 PPAR α is involved in the duration of LTB₄ and arachidonic acid-mediated inflammation. Ear thickness due to application of *a*, arachidonic acid; *b*, leukotriene B₄; and *c*, TPA was measured over time in wild-type (+/+) and PPAR α -knockout (-/-) mice²⁷. Treatment with arachidonic acid and LTB₄ resulted in prolonged inflammation in (-/-) mice, whereas the response to TPA was comparable in both mouse types. An asterisk indicates that the difference is statistically significant ($P < 0.03$).

reproduce normally. They still have peroxisomes and basal constitutive expression of the genes involved in fatty acid metabolism, but when challenged with the hypolipidaemic agents Wy14,643 or clofibrate, they are unable to increase the expression of these genes²⁷. This defect correlates with the increased duration of LTB₄-mediated inflammation. As PPAR α knockout mice eventually manage to control the inflammation, LTB₄ must presumably be slowly cleared from the site of inflammation, either through basal ω - and β -oxidation or by an alternative pathway. Together these findings suggest that PPAR α senses LTB₄ levels and down-regulates them by increasing its oxidative degradation.

PPAR α as director of adaptive responses

Organisms rely on the inducible ω - and β -oxidation pathways for the control of fatty acid levels, inflammation and detoxification of various xenobiotics (peroxisome proliferators). These adaptive responses depend on how effectively PPAR α , which regulates the amount of ω - and β -oxidation, senses the presence of activating molecules. PPAR α stimulates target-gene expression in response to a range of signals, including arachidonic acid and its metabolites, other PUFAs, phthalates, 5,8,11,14-eicosatetraynoic acid (ETYA), fibrates and xenobiotics^{11,39}. Although many of these activators have been characterized, until now no ligand for PPAR α has been found. The binding and activation of PPAR α by the structurally different ligands Wy14,643 and LTB₄ results in induction of the same pathways (ω - and β -oxidation). The role of this activation might vary according to its site or physiological context. PPAR α is expressed in the liver, kidney, brown adipose tissue, eye, digestive tract, immune and genital systems²⁸, but its function is known only in the liver (catabolism of fatty acids and xenobiotics) and the immune system (determination of the dura-

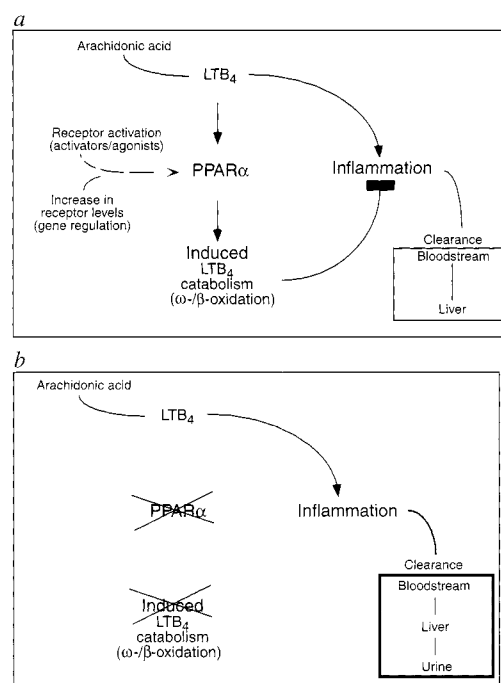


FIG. 6 The role of PPAR α at the site of inflammation. *a*, Inflammation control by PPAR α . LTB₄ is a powerful chemotactic agent of inflammation. Control of the duration of the inflammatory response is mediated by direct interaction between LTB₄ and PPAR α . LTB₄ acts as a ligand and activator of PPAR α to induce transcription of enzymes involved in ω - and β -oxidation. As a result, PPAR α induces the catabolism of LTB₄ to its less potent metabolites. Thus PPAR α activity modulates the duration of inflammation by controlling the turnover of LTB₄. Anti-inflammatory agents that can be envisaged to act via this pathway would be agents that induce or enhance PPAR α activity. PPAR α can be activated by polyunsaturated fatty acids, arachidonic acid metabolites, clofibrate, Wy14,643 and Iloprost^{14-17,39}. Glucocorticoids such as dexamethasone induce the expression of PPAR α ³³, suggesting that one feature of their anti-inflammatory effect is mediated by PPAR α . Alternatively, synthetic agonists/ligands of PPAR α could be anti-inflammatory reagents. *b*, Consequences of a non-functional PPAR α . PPAR α can be disabled either totally, as in a knockout mouse, or temporarily, in the presence of a PPAR α antagonist. As PPAR α induces the catabolism of LTB₄, the absence of PPAR α is equivalent to a metabolically more stable environment for LTB₄. In this case, the turnover of LTB₄ is primarily determined by the basal ω - and β -oxidation activities and clearance of LTB₄ by the bloodstream: the net result is a prolongation of inflammation.

tion of an inflammatory response). Presumably, to be an efficient director of adaptive processes, PPAR α needs to bind a broad array of ligands, so the success of PPAR α is inherent in its promiscuity.

The triglyceride-lowering effect of the synthetic PPAR α ligand Wy14,643 is linked by our findings with transcriptional control of the overall lipid metabolism. PPAR α regulates the activity of several genes whose products are associated with the entire oxidative pathway, including the uptake, activation and oxidation (microsomal, peroxisomal and mitochondrial) of fatty acids, as well as ketogenesis¹¹. Thus the effects of the hypolipidaemic drug are probably not attributable to a single event: instead it results from activation of the transcription factor PPAR α , which in turn modulates the entire oxidative pathway at several levels.

The identification of LTB₄ as a ligand of PPAR α is important as it reveals a key function for PPAR α in inflammation. We propose a pathway that confers tight control over the activity of LTB₄ during an inflammatory response via regulation of the turnover of this potent chemotactic lipid mediator (Fig. 6). This pathway involves a feedback mechanism in which LTB₄ induces its own catabolism by activating the transcription factor PPAR α . Thus, compounds that enhance PPAR α function, by either increasing receptor levels or activating the receptor, should be anti-inflammatory (Fig. 6a). Given the promiscuous nature of

PPAR α , a variety of fatty acids and eicosanoids could regulate their own inactivation or clearance through activation of PPAR α . Conversely, blocking PPAR α function by either a receptor antagonist or gene disruption should extend the inflammation (Fig. 6b). Consistent with this model, preliminary experiments indicate that when administered intravenously, LTB $_4$ is cleared from the plasma of PPAR α (-/-) mice less efficiently than of (+/+) mice (data not shown).

A transcription factor that controls the half-life of a potent inflammation mediator has therapeutic potential. The PPAR α nuclear hormone receptor has activities that are very different from those of the membrane-bound LTB $_4$ receptor, a postulated seven-transmembrane-spanning receptor that remains to be cloned and characterized. This membrane receptor, which is also expressed in cells of the immune system, is the primary target of LTB $_4$, initiating chemotaxis and inflammation once bound. PPAR α controls the availability of this lipid signalling molecule, an arachidonic acid metabolite, for its membrane receptor. Although the nuclear PPAR α and the membrane LTB $_4$ receptors share the same ligand, they control opposing biological effects, so crosstalk between these receptors is critical for the success of metabolite-controlled lipid signalling in maintaining homeostasis.

Other potential ligands of PPAR α can be tested from among the many activators of the receptor. The neutrophil chemotractor 8S-HETE (an arachidonic acid metabolite)⁴⁰ and the immunomodulator DHEA-S (an endogenous steroid)⁴¹ are two such signalling molecules that mediate gene induction through PPAR α . The arachidonic acid metabolites LTB $_5$ (ref. 42) and 12-HETE⁴³ are endogenous compounds that are also implicated in the inflammatory response. Furthermore, many other membrane LTB $_4$ -receptor effectors might also be ligands of PPAR α —the banks of synthetic membrane LTB $_4$ receptor antagonists and agonists that have been developed over the years need to be re-evaluated for the presence of PPAR α ligands.

The identification of ligands for PPAR α will help to clarify the mechanisms involved in the control of lipid metabolism. Many lipid-related disorders result from alterations in the balance between lipid storage or production and degradation by oxidative catabolism. Our findings suggest that PPAR α is a promising target for drug intervention in seemingly different disorders such as obesity, hyperlipidaemia and cardiovascular disease, and inflammatory conditions like rheumatoid arthritis, lupus and psoriasis. □

Methods

Transfection assays. 2.5×10^5 HeLa cells per well in 6-well plates were transfected with expression (1 μ g) and reporter plasmids (1.5 μ g) using calcium phosphate precipitation¹⁸. An internal control reporter plasmid (2 μ g) expressing the luciferase gene was used to standardize the transfection efficiency. Transfected cells were incubated with either activator (LTB $_4$ (Cascade Biochemicals), BRL49653 (Glaxo-Wellcome), Iloprost (Schering)) or solvent (dimethylsulphoxide or ethanol). CAT assays were normalized using luciferase activity as internal standard. Expression plasmids for full-length wild-type PPAR (α , β and γ) were based on the pSG5 vector (Stratagene). Reporter plasmids were derived from the parent pBL-CAT8⁺ vector and were constructed as described⁴⁴.

Primary hepatocyte culture. Male Sprague-Dawley rats (BRL, Basel) weighing 200–250 g were anaesthetized with pentobarbital and their livers were perfused *in situ* with collagenase. Preparation of hepatocytes before plating was done as described³³. The hepatocytes were cultured in monolayer (1.5×10^5 cells cm $^{-2}$) in Williams' E medium (Gibco, BRL) supplemented with 5% charcoal-treated fetal calf serum and antibiotics, at 37°C in a humidified atmosphere of 5% CO $_2$ /95% air. LTB $_4$ (Cascade Biochem), Wy14,643 (Campco Scientific) or solvent (control), were added immediately after seeding the cells. RNase protection experiments were done as before with probes for ACO and L27 mRNAs³³. Results were quantified on a phosphorimager (BioRad), and the amounts of ACO mRNAs normalized with respect to L27 ribosomal protein mRNA, the levels of which are not modified by activated PPAR α .

Fusion-protein expression. The xPPAR α (LBD) fragment was amplified by PCR and cloned into pGEX vectors (Pharmacia). The GST-xPPAR α (LBD) fusion protein was expressed in *E. coli* BL2 DE3 (pLysS) bacteria. Cells were lysed by repeated freeze-thawing, this DNA removed by centrifugation followed by incubation with DNase I. Fusion proteins were purified by glutathione-sepharose affinity chromatography (Pharmacia) followed by Sephadex size-exclusion chromatography (Pharmacia). Protein concentrations were determined by the BioRad protein assay using γ -globulin as standard.

Ligand-binding reactions. 3 H-LTB $_4$ (specific activity, 207 Ci mmol $^{-1}$; Amersham) and 10 μ g total protein were incubated for 3.5 h (on ice and in the dark) in a 50- μ l reaction containing 50 mM Tris, 10 mM MgCl $_2$ and 100 mM NaCl (pH 8.0). Bound ligand was separated on a NAP-5 column (Pharmacia) using a buffer containing 10 mM HEPES, 1 mM EDTA, 50 mM NaCl, 5% glycerol and 0.05% Triton-X 100 (pH 7.4). The bound fraction was quantified by scintillation counting. In the competition experiments, 3 H-LTB $_4$ was kept at 50 nM and different amounts of non-radioactive competitor were added.

Inflammation tests. The mouse-ear inflammation tests were done as described³⁴. Five female mice (pure-bred Sv/129 strain, 7–8 weeks old, either +/- or -/- for PPAR α) were used for each treatment group. For each mouse, test substance (2 mg arachidonic acid in ethanol, 5 μ g LTB $_4$ in dimethylsulphoxide (stored at -20°C under argon, applied immediately) or 2 μ g of TPA in ethanol) was topically applied to the right ear and the solvent carrier to the left. Animals were lightly anaesthetised with 2.5% avertin before application of compounds. Ear thickness, measured with a micrometre gauge was recorded at different times. Mean ear swelling (%) was taken as the test ear thickness divided by the control ear thickness $\times 100$.

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Detection of high-velocity ^{26}Al towards the Galactic Centre

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THEORY predicts¹ that radioactive ^{26}Al (which has a half-life of 0.72 Myr) is released into the interstellar medium by nova and supernova explosions, from the winds of massive stars in the Wolf-Rayet phase, and from less-massive giant stars in very late stages of the asymptotic giant branch phase. Observations of 1,809-keV γ -ray emission line from ^{26}Al can therefore be used as a tracer of Galactic nucleosynthesis during the past million years^{2,3}. The irregularity of the emission in the plane of the Galaxy⁴⁻⁷ suggests that the dominant sources are likely to be massive stars and supernovae; the other predicted sources are older, and therefore expected to be distributed more uniformly. Here we report the detection of the 1,809-keV emission line from the direction of the Galactic Centre, and we show that the line width is approximately three times that expected^{8,9} from the effect of Doppler broadening due to Galactic rotation. The high velocities inferred from the line width favour an origin of the ^{26}Al in supernovae or Wolf-Rayet stars. Moreover, the fact that the ^{26}Al has maintained such high velocities is difficult to reconcile with our current understanding of the propagation of material in the interstellar medium.

The Gamma Ray Imaging Spectrometer (GRIS)¹⁰ is a balloon-borne high-resolution γ -ray spectrometer consisting of an array of seven germanium detectors surrounded by a thick (15 cm) active NaI anticoincidence shield. This instrument is one of the most sensitive high-resolution γ -ray spectrometers, as shown by results from previous flights such as the observations of the Galactic Centre 511-keV positron annihilation line¹¹ and the SN1987A ^{56}Co lines¹². GRIS has been recently reconfigured with a wide-field collimator (field of view $100^\circ \times 75^\circ$ full-width at half-maximum, FWHM), and the 15-cm-thick NaI blocking crystal to optimize its capability for observation of diffuse γ -ray sources such as the cosmic diffuse background and Galactic ^{26}Al emissions. The measurements reported here were made on a flight from Alice Springs, Australia, on 24–26 October 1995. The total germanium detector area and volume were 237 cm² and 1,647 cm³ respectively. The duration at float altitude was 32 hours at an average atmospheric depth of 3.8 g cm⁻². The collimator was almost pointed at the zenith. From Alice Springs, the Galactic Centre, south Galactic pole and Galactic plane (Galactic longitude $l = 240^\circ$) transit nearly overhead (see top of Fig. 1). GRIS observed alternating 10-minute exposures with the blocking crystal open and closed.

The variation of the 1,809-keV line intensity during the flight is shown in Fig. 1. These values include the instrumental background line due to interactions of cosmic-ray-induced neutrons with Al in the instrument ($^{27}\text{Al}(n, np)^{26}\text{Mg}^*$ and $^{27}\text{Al}(n, 2p)^{26}\text{Na}(\beta^-)^{26}\text{Mg}^*$). Both the Galactic line and the background line are included in the model we have fitted to the data. The variation of the rates with time clearly shows an excess at the Galactic Centre and remains constant during the Galactic pole and Galactic plane transits. The excess is detected independently in each of the six operating Ge detectors, as expected from a real source detection. With a 100° field of view, the net flux is dependent on the distribution of ^{26}Al

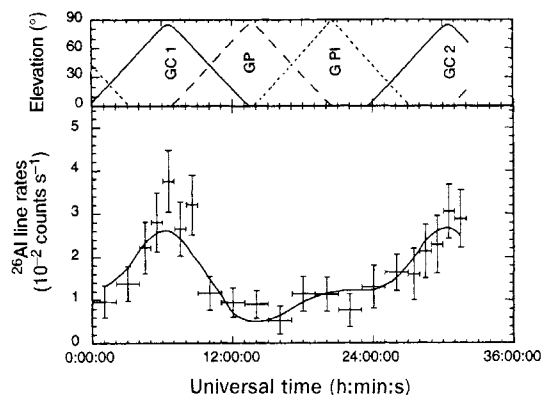


FIG. 1 Evolution of the measured 1,809-keV line rates during the 25 October 1995 GRIS flight. The data include both astrophysical and background lines. The elevation angles of the different targets are shown at the top (GC 1, Galactic Centre, 1st transit; GP, south Galactic pole; G PI, Galactic plane at $l = 240^\circ$; and GC 2 Galactic Centre 2nd transit). The count rates increase by a factor of three at the two Galactic Centre transits, providing a clear detection of Galactic ^{26}Al . Using a model derived from the Galactic γ -ray distribution (>70 MeV) measured¹³ by COS-B (solid line), we obtained a flux of $(4.8 \pm 0.7) \times 10^{-4}$ photons s⁻¹ cm⁻² rad⁻¹ (6.8 σ level). The horizontal axis gives time elapsed after the start of the measurements.

emission. Using a model derived from the COS-B satellite measurement of the Galactic γ -ray distribution (>70 MeV)¹³, which has been used to fit most previous results^{4,14-20}, and taking into account the GRIS instrument response plus the effects of atmospheric absorption, we obtained a flux of $(4.8 \pm 0.7) \times 10^{-4}$ photons s⁻¹ cm⁻² rad⁻¹. This value is compatible with previous measurements and yields a detection at the 6.8 σ level.

The measured astrophysical 1,809-keV line spectrum is given in Fig. 2c. It was derived by subtracting the Galactic pole and Galactic plane accumulation (Fig. 2b) from the sum of both Galactic centre transits (Fig. 2a). As the line rates observed at the Galactic pole and the Galactic plane transits are very similar (Fig. 1), and we do not expect significant 1,809-keV line emission from the Galactic pole region, the observed line in Fig. 2b should be mostly of instrumental origin. The spectrum was fitted with a gaussian profile model. The resulting astrophysical line centroid at $1,809.0 \pm 0.6$ keV is consistent with ^{26}Al rest energy of $1,808.65 \pm 0.07$ keV. The width of the ^{26}Al line is the most remarkable result. The measured width (6.4 (+1.2, -1.1) keV FWHM) is the quadratic sum of the instrument resolution and the intrinsic width. The instrument energy resolution was precisely determined by fitting a line to the measured widths of many intense narrow lines in the background spectrum. The energy resolution at 1,809 keV is 3.41 ± 0.1 keV, which implies that the intrinsic width of the astrophysical line is 5.4 (+1.4, -1.3) keV FWHM.

We investigated the potential sources of systematic error that might generate such a broad feature in the GRIS spectrum. The instrumental background spectrum in this energy range contains a number of narrow lines, including the one at 1,809 keV (see Fig. 2a, b). The background line at 1,779 keV results from neutron capture, $^{27}\text{Al}(n, \gamma)^{28}\text{Al}$, followed by β -decay (mean life 3.23 min) to ^{28}Si . We note that both the 1,779- and 1,809-keV background lines originate mainly from the interaction of neutrons in aluminum. Thus, systematic errors in the data analysis that could simulate a broad line or a net cosmic flux should give similar results for both lines. The 1,779- and 1,809-keV lines were both analysed for the data corresponding to the four transits indicated in Fig. 1. A model consisting of a gaussian plus a power law was fitted to each line plus surrounding continuum. The resulting best-fit line para-

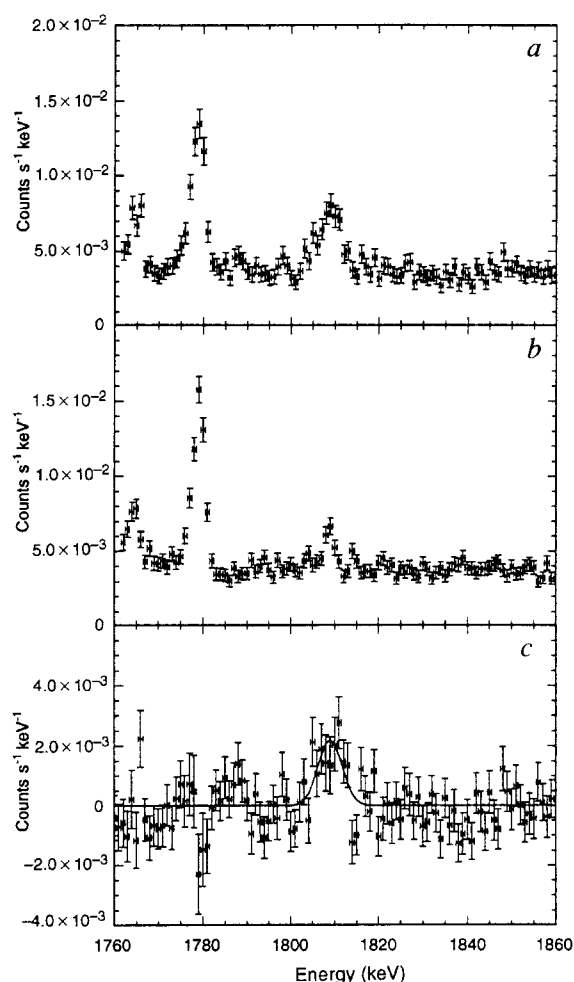


FIG. 2 γ -ray count rate spectrum near 1,809 keV. *a* and *b* are the accumulations at both Galactic Centre transits and at the Galactic pole and Galactic plane transits. Each channel has a width of 1 keV. These spectra show background line features at 1,764 keV from the decay chain of ^{238}U , 1,779 keV from the de-excitation of ^{28}Si , and 1,809 keV from the de-excitation of ^{26}Mg . The net Galactic γ -ray spectrum, derived by subtracting spectrum *b* from spectrum *a*, is displayed in *c*. It shows line emission at only 1,809 keV produced in the decay of interstellar ^{26}Al . There is a small residual ($\sim 2\sigma$) at the location of the 1,779 keV background line that is probably due to a combination of small changes in the background line intensity and small gain shifts. Because the signal-to-background ratio for the 1,809 keV line is large (a factor of ~ 2) the effect on the 1,809 keV intensity will be small ($<4\%$). This effect is included in the systematic error estimate. The solid curve is the best fit of the data to a gaussian line shape. Assuming an instrument energy resolution of 3.4 keV, the intrinsic width of the astrophysical line is $5.4 (+1.4, -1.3)$ keV FWHM, which is more than three times the values expected from previous theories. The significance of this measurement allows one to exclude a line width merely reflecting Galactic rotation (that is, 1.8 keV) within 97% probability.

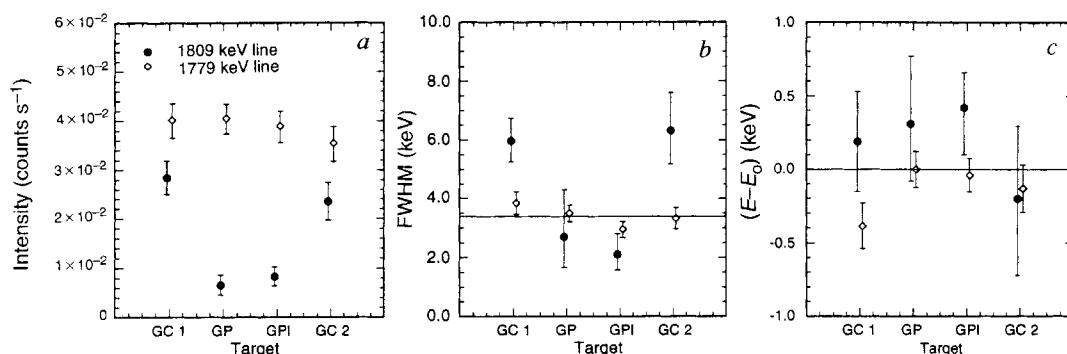
meters are shown in Fig. 3. The 1,809-keV line intensity (see Fig. 3*a*) increases by a factor of three at the Galactic Centre transits while the 1,779-keV line intensity remains unchanged, within the statistics, during the whole flight. This indicates that the 1,809-keV line excess observed at the Galactic Centre transits is not instrumental in origin. The width of the lines shows a similar behaviour (see Fig. 3*b*). The 1,809-keV line gets broader in both Galactic Centre transits and is consistent with a narrow line for both the Galactic pole and Galactic plane transits. The 1,779-keV line width remains practically unchanged for the four targets, indicating that the broad nature of the 1,809-keV line excess observed

at the Galactic Centre transits is not instrumental. Last, the position of the centroid with respect to the line rest energy does not vary significantly with the target for either of the lines (see Fig. 3*c*), demonstrating that the quality of the gain correction does not change during the flight. We have estimated a conservative upper limit on the additional line broadening produced by systematic effects to be <0.5 keV. This limit was derived by assuming that the 1,779-keV line parameter fluctuations (Fig. 3) were not of statistical origin and reflected real changes in the instrumental performance.

The comparison of our present result with past observations is inconclusive, largely because of the low significance of previous line-width determinations. The width reported by the high-resolution spectrometer HEAO-C¹⁴ (with a 43° field of view) was compatible with a narrow line, giving a 1σ intrinsic-width upper limit of 3 keV. Other high-resolution instruments^{16,20} (with a $\sim 20^\circ$ field of view) have also reported a line width compatible with a narrow line. An important exception is the previous GRIS measurement with a 20° field of view. The 1,809-keV line was only detected at 2.5σ significance but the width observed was 5.1 ± 2.2 keV FWHM. SMM¹⁵ and COMPTEL⁴ have made significant detections of this line, but the modest energy resolution of these instruments (based on scintillator detectors) does not constrain the width. A mode consisting of diffuse broad emission plus narrow emission concentrated at the Galactic plane could induce a line broadening dependent on the field of view of the instrument. However, the flux detected by GRIS does not reveal the existence of extra flux invisible to the narrow-field instruments. Also, a single gaussian model provides the best fit to the observed feature.

The detection of such a wide line has very important implications for the origin of Galactic ^{26}Al . The 1,809-keV line was expected to have a width of <1.8 keV FWHM: because of its long lifetime, ^{26}Al should have slowed down before decaying and it should therefore exhibit velocities typical of differential Galactic rotation (that is, $<150 \text{ km s}^{-1}$) plus random motions of the interstellar medium^{8,9}. The measured line broadening is a factor of three larger than predicted and implies that the ^{26}Al decay occurs at velocities $>450 \text{ km s}^{-1}$. Only novae, supernovae and Wolf-Rayet stars are able to release this isotope at such high velocities. Thus without reheating or acceleration, asymptotic giant branch (AGB) stars are not likely to be major contributors to the production of the Galactic ^{26}Al . Even with high initial velocities ($>1,000 \text{ km s}^{-1}$), it is difficult to understand how ^{26}Al could maintain these speeds for 10^6 years. High-velocity material in the Galactic plane should be slowed down by interactions with the interstellar medium on timescales of less than 10^5 years (ref. 3). New scenarios are required to account for such a broad line. One possibility is the broadening due to the contribution of local sources. Material ejected from local sources could contain freshly synthesized ^{26}Al ($<10^5$ years old) that has not yet slowed down. In the Galactic Centre direction, the closest source of this kind is Loop I. This radio structure, a residual of nearby supernova activity, is centred on the Sco-Cen association, has an angular diameter of 116° and its centre is at a distance of 170 pc from the Sun²¹. Although this source could emit the broad line, the predicted flux is ~ 20 times too low to account for our observation⁶. An alternative explanation is that part of the ejecta could escape the high-density disk before stopping. Such a phenomenon occurs in galactic 'chimneys', which are cavities in the interstellar medium created by multiple supernova explosions that act as conduits for the efficient transport of hot gas from the galaxy's disk to its halo²². If these objects were very common in the Galaxy (that is, they were typical features of clusters of massive stars) they could explain the observed line broadening. Such a scenario has been alluded to as a possible explanation for the origin of dark matter in the Galactic halo^{22,23}. A third possibility is ^{26}Al incorporated into high-velocity dust grains N. Pranztos, personal communication). From the observations of SN1987A, we learned that high-velocity grains form in the ejecta within a couple of years of the explosion²⁴. These grains with a low charge/mass ratio could be

FIG. 3 Evolution of the intensity, width and centroid energy of the 1,809- and 1,779-keV line features from the spectra at the transits of the targets described in Fig. 1. (E, line centroid; E_0 , theoretical energy.) Owing to the similar origin of both background lines, systematic errors in the data analysis that could simulate a broad line or a net cosmic flux should give the same result for both lines. The intensity (a) and width (b) of the 1,809-keV line increase dramatically at the Galactic Centre transits while the 1,779-keV background line remains unchanged. This shows that the 1,809-keV line excess and broadening observed at the Galactic Centre transits is of external origin. The energy of the lines (c) does not vary significantly, demonstrating that the gain correction was properly performed. A con-



only weakly coupled to the ejecta plasma and may not be stopped at the outer shock boundary of the plasma, continuing into the interstellar medium where they might survive in the hot phase for 10^6 years before being slowed down or destroyed. Unfortunately, the modest angular resolution of the GRIS instrument does not allow us to determine whether the broad line emission comes primarily from sources above the Galactic plane (and therefore attributable to chimneys), or mainly from within it.

Progress on understanding the origin of ^{26}Al requires improved observations performed with instruments combining good sensitivity with high angular and energy resolution. Measurements of the line profile and Doppler shifts due to Galactic rotation in different directions would provide valuable information about the location⁹ and properties of the ^{26}Al sources. A more sensitive detector would also allow confirmation of the existence of ^{60}Fe line emission, which has been predicted²⁵ to be distributed in a similar way as the ^{26}Al emission if supernovae were the major source of ^{26}Al . The next generation of γ -ray spectrometers (for example, the INTEGRAL Spectrometer²⁶) should be able to address these issues, thereby providing the most accurate information about the current nucleosynthetic activity in our Galaxy. □

servative upper limit of <0.5 keV was estimated for the line broadening induced by possible systematic errors. This limit was derived by assuming that the intensity, width and centroid fluctuations of the 1,779-keV line were not of statistical origin and reflected real changes in the instrument performance.

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Secular changes in the xenon and krypton abundances in the solar wind recorded in single lunar grains

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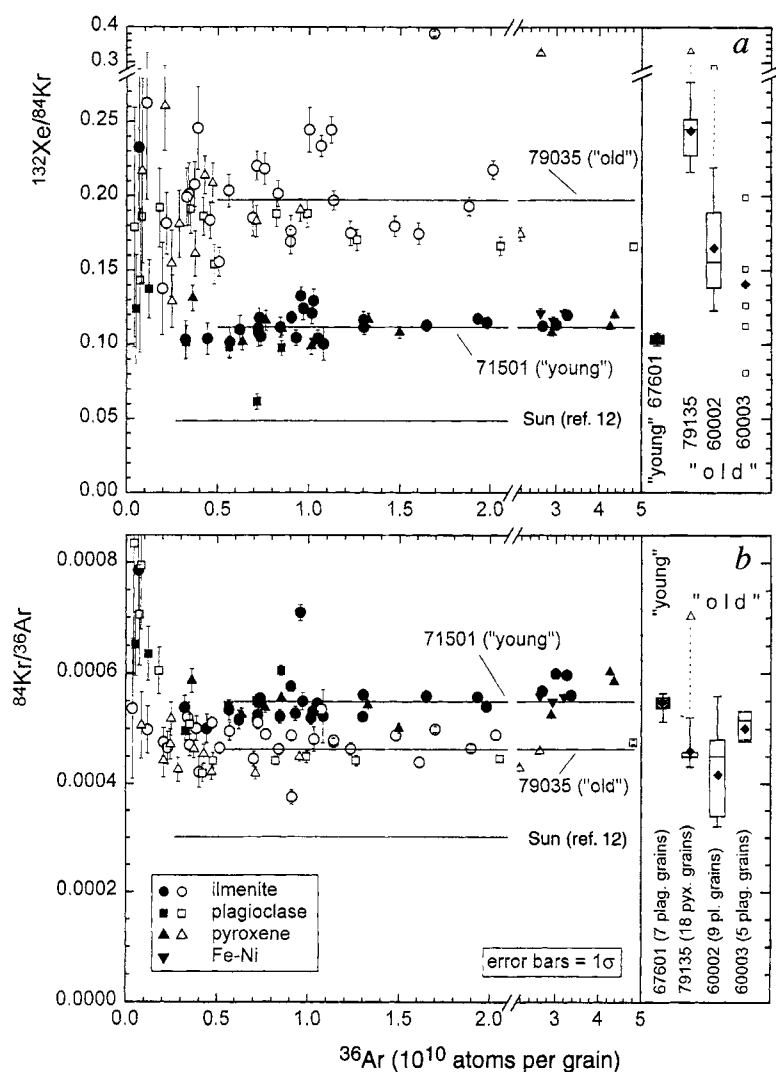
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NOBLE gases implanted into the lunar surface trace elemental and isotopic abundances in the solar wind during the Sun's lifetime, and so potentially provide a valuable record of changing physical processes on the Sun. But the interpretation of this record is not straightforward, as it has proved difficult to discriminate the effects of solar variations from changes due to subsequent alteration processes on the lunar surface. Here we report analyses of krypton and xenon abundances in individual grains of lunar soil (in contrast to the multi-grain samples used previously), which permit such discrimination. The abundances of the heavy noble gases have not been altered on the Moon, confirming the view¹ that there have been modest variations in the composition of solar wind, on timescales of 10^8 – 10^9 years, during the past several billion years. Moreover, the measured enrichment of xenon, relative to argon, is similar to a well-known excess of easily ionizable elements in the solar chromosphere. As xenon is not an easily ionizable element, this finding supports the hypothesis that the selection effect arises from element-specific ionization times in the solar chromosphere^{2,3}.

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FIG. 1 $^{132}\text{Xe}/^{84}\text{Kr}$ (panel a) and $^{84}\text{Kr}/^{36}\text{Ar}$ (panel b) ratios of the implanted solar component (solar wind plus solar energetic particles) in ~ 160 single grains of six lunar soils. Data are blank-corrected (blanks are constant to within $\pm 40\%$ at $\sim 15,000$, $\sim 60,000$ and $\sim 8 \times 10^5$ atoms of ^{132}Xe , ^{84}Kr and ^{36}Ar , respectively). 'Young' samples that received their solar gases within the past ~ 50 – 100 million years are distinguished from 'old' samples that were irradiated some 1,000–2,000 million years ago. For one young and one old sample each, the individual data points are given, with the solar ^{36}Ar amount on the abscissa. Multi-grain aliquots of these samples have been analysed by on-line etching^{5,6}. Data from the four other samples are shown in box charts at the right-hand side of each panel (elemental ratios of the middle 50% of the grains fall within the boxes, the other half of the grains plot within the error-bar-like extensions; horizontal lines in the boxes indicate the median, filled diamonds the mean). Three outliers as well as all five data points from soil 60003 in a are shown individually. Only grains with more than 0.5×10^{10} atoms of ^{36}Ar are considered in the box charts, because grains with less gas are measured rather imprecisely, as can be seen in the left-hand plots. The average ratios of all grains with $^{36}\text{Ar} > 0.5 \times 10^{10}$ atoms of the two samples on the left are shown as horizontal lines, as are the ratios inferred for the Sun¹². Xe/Kr and Kr/Ar values in grains of various mineral types from soil 71501 are nearly constant ($\pm 9\%$, with only one precisely measured grain forming an exception in each ratio, probably reflecting experimental artefacts), but clearly different from the value in the Sun. The two mean ratios are identical to those obtained by on-line etching^{5,6}. Data of the other young sample (67601) are also uniform in all precisely measured (plagioclase) grains and the mean is in excellent agreement with that of 71501. The Xe/Kr ratios in grains from 79035 scatter considerably and are on average another factor of 2 higher than the values for 71501. The data show a secular change of the Kr/Ar and the Xe/Kr ratios in the solar corpuscular radiation in opposite directions. The Xe/Kr scatter in 79035 indicates that different grains in this sample were irradiated at the lunar surface at rather variable times. The other old samples confirm this interpretation.



No direct information on the elemental abundances of the heavy noble gases in the solar wind is available, and it has proved difficult to determine whether lunar samples truly record these values. One approach is to determine noble-gas concentrations as a function of depth within lunar soil grains. The conventional method of stepwise gas release at increasing temperature has failed in this respect, because lighter gases leave the grains at lower temperature than heavier ones. Stepwise etching by HF at room temperature in an ultra-high-vacuum device connected to a mass spectrometer allowed us to determine depth profiles of noble-gas concentrations in grains^{4–6}. Results from thousands of lunar grains are combined in such measurements. An alternative approach is to analyse the noble gases in single grains. Here we report such measurements for the particularly rare gases Kr and Xe. Although depth information is lost with this technique, the grain-by-grain noble-gas record should allow us to distinguish abundance variations occurring at the solar surface from later effects on the Moon. We studied ilmenite, pyroxene, plagioclase and Fe–Ni grains, minerals which retain the light gases He and Ne to widely different degrees. Most of the grains contain fewer than 700,000 ^{132}Xe atoms and thus require a system with very low background and high sensitivity^{7,8}. The ~ 200 - μm grains were melted with an infrared laser beam such that only the analysed grain was heated substantially. Our mass spectrometer counts one Xe ion per second for every 16,000 atoms in the system. Two of the six samples studied, soils 71501 and 67601, were taken from within the uppermost few centimetres of the present-day lunar surface and mostly contain grains which trapped their solar gases within

the past 100 and 50 million years, respectively. The other samples were exposed on the lunar surface roughly 1,000–2,000 million years ago, 79035 and 79135 are compacted soil breccias, soils 60002 and 60003 were recovered from some 2 m depth. We will call the two groups 'young' and 'old' samples, respectively.

The etch data^{4–6} proved that a solar-wind component with an isotopic composition of He–Ar essentially identical to that trapped in foils during the Apollo lunar missions⁹ is present in the outermost few hundred ångströms of lunar grains. At larger depths we found a solar energetic particle component, which is enriched relative to the solar wind in the heavier isotopes, but has similar elemental abundances as the solar wind. Ar/He and Ar/Ne ratios in the first etch steps are much above the present-day solar-wind values⁹, but approach this reference composition deeper in the grains; Kr/Ar and Xe/Kr ratios are constant, but differ for samples that were irradiated by the Sun at different times. We inferred that the well known He and Ne losses^{10,11} affect almost exclusively the low-energy solar-wind component and that Ar, Kr and Xe are retained unaltered at all depths. If so, we would expect uniform Kr/Ar and Xe/Kr ratios in all grains of a given soil, at least if they all trapped their solar gas at about the same time. Otherwise, if the heavy noble-gas abundances were altered on the lunar surface, we would expect grain-to-grain differences, perhaps as a function of gas concentration.

For two samples we show in Fig. 1 the $^{84}\text{Kr}/^{36}\text{Ar}$ and $^{132}\text{Xe}/^{84}\text{Kr}$ ratios of all grains individually, with the ^{36}Ar concentration on the abscissa. On the right-hand side of each panel, the pooled data for each of the four other samples are shown. Nearly all grains from

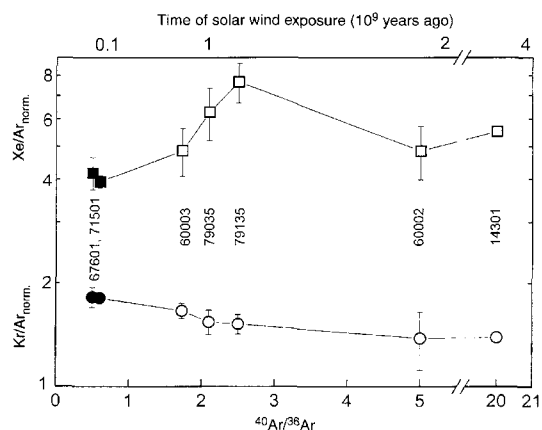


FIG. 2 Kr/Ar and Xe/Ar ratios in solar corpuscular radiation as deduced in this work normalized to the respective ratios in the Sun¹² versus the mean $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of each sample. The latter is a proxy for when a sample was exposed to the solar wind at the lunar surface²⁹, because the flux of radiogenic ^{40}Ar escaping from the Moon and being reimplanted into grain surfaces has decreased by more than a factor of ten during lunar history. This semiquantitative surface-irradiation age scale is indicated on the top abscissa. Because the solar photosphere can be assumed to have bulk solar Kr and Xe abundances, the figure thus shows the temporal variation of Kr-Ar and Xe-Ar separation factors between corona and photosphere. Besides the single-grain data of this work, the on-line etch data of very old soil breccia 14301 are shown⁶. Error bars represent the standard deviation of each single-grain data set (no errors given for 14301). These are conservative uncertainties (standard errors of the mean are several times lower) in the sense that for the old samples the spread in the data is probably largely due to a scatter of the surface irradiation age for individual grains around the mean abscissa values plotted. Determinations of the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in single grains, preferentially such with little *in situ* produced radiogenic ^{40}Ar as the very rare Fe-Ni particles, are needed to quantify this spread in irradiation age.

the young soil 71501 have a constant Xe/Kr ratio within 2σ uncertainties (Fig. 1a), particularly well displayed for the 16 most gas-rich, and thus most precisely measured, grains. The mean Xe/Kr ratio is 2.3 times above the inferred value in the Sun¹², which is deduced by interpolating meteoritic abundances of elements adjacent to the noble gases in the periodic table. A change in the Xe/Kr ratio of this size during or after implantation would hardly be so constant for all grains, especially since the heavy noble-gas concentrations vary by about an order of magnitude. This expectation is corroborated by the striking contrast between the uniform Xe/Kr values on the one hand, and the very variable Ar/He and Ar/Ne ratios in the same grains on the other. The latter are up to ~ 100 times higher for He-Ne-leaky plagioclase than for ilmenite and Fe-Ni (data not shown). The single-grain data therefore reinforce the statement⁶ that mineral grains from soil 71501 conserve the average relative Xe and Kr abundances in the solar corpuscular radiation (solar wind plus solar energetic particles) in the past ~ 100 million years. This conclusion is further strengthened by data from the other young sample, 67601 (Fig. 1a).

The Xe/Kr patterns of the four old samples in Fig. 1a differ in two respects from those of the more recently irradiated ones. First, the 'old' 79035 grains plot above the 'young' 71501 grains, on average by nearly a factor of two, as expected from on-line etch data^{5,6} and bulk sample analyses^{1,13}; also, the other old samples have higher Xe/Kr ratios than the young ones. Second, the data points of the old samples scatter in most cases considerably more than those of the young ones. We therefore have to discuss whether Xe/Kr ratios in grains that were irradiated a long time ago are altered from their true value as recorded by young grains. If so, the conclusion¹⁶ of a secular variation of the Kr and Xe abundances in the solar corpuscular radiation would be difficult to defend. Two arguments speak against such a diffusive alteration

on the Moon. First, pyroxene and plagioclase grains would be expected to have higher Xe/Kr ratios than ilmenites, because the former minerals have up to 100 times higher Ar/He and Ar/Ne ratios. Figure 1a shows no such systematic differences between plagioclase, pyroxene and ilmenite. An even more striking argument is provided by the data shown in Fig. 1b. Gas losses that change the Xe/Kr ratio to such a variable extent should also change the Kr/Ar ratio: grains that lost more Kr than Xe should also have lost more Ar than Kr. This is not the case. With very few exceptions, Kr/Ar ratios are nearly uniform in all grains of all old samples and the mean values are about 10–20% lower than the 71501/67601 average. Figure 1a therefore strongly suggests that a temporal variability of the Xe/Kr ratio in the solar corpuscular radiation is recorded even in single grains from any given old sample. In addition, as well as providing strong evidence that the Xe/Kr variations are not induced on the Moon, Fig. 1b shows that the old samples recorded a somewhat lower Kr/Ar ratio in the solar corpuscular radiation than the young samples. This secular trend runs opposite to that for Xe/Kr.

Apart from the noble gases, only the highly volatile elements H, C and N are sufficiently depleted in lunar material that a solar contribution can possibly be detected. In particular, a debate has been going on for many years whether or not nitrogen in lunar soils was predominantly implanted by the solar wind^{14–17}. This is an important unsolved problem, because a solar origin for this nitrogen would imply that the $^{15}\text{N}/^{14}\text{N}$ ratio in the solar wind varied by $\sim 30\%$ over the past several thousand million years^{18,14}, a huge change which is very difficult to explain. The question is crucial here because the $^{14}\text{N}/^{36}\text{Ar}$ ratio in lunar samples is about 10 times higher than both the value in the Sun¹² and that in solar energetic particles measured by spacecraft¹⁹. Hence, if N in lunar soils is mostly solar, the conclusion reached here would require that Ar, Kr, and Xe were all depleted on the Moon by a constant factor relative to N. We cannot propose a convincing way to achieve this; we therefore think that our data provide evidence for a major non-solar N component in lunar soils (for example, lunar indigenous nitrogen¹⁶ or terrestrial nitrogen²⁰), although the secular $^{15}\text{N}/^{14}\text{N}$ variability is also difficult to explain by such sources¹⁴. Crucial experiments to elucidate the origin of nitrogen in lunar soils would be simultaneous nitrogen and noble-gas analyses in single grains and in multi-grain samples with variable surface layers removed by etching, as well as new generations of space-borne solar-wind collectors and analysers that also record the heavy noble gases and nitrogen.

Elements with a first ionization potential (FIP) $< 10\text{ eV}$ are enriched in the solar corpuscular radiation by a factor of about four^{21,22}. This 'FIP-effect' is not well understood but is presumably due to a separation of ions from neutral particles in the chromosphere²³. The single-grain data now show that Xe is overabundant relative to Ar by a very similar factor as are low-FIP elements, while Kr is partly enriched. This is remarkable, as both Xe and Kr have a FIP above the threshold of $\sim 11.2\text{ eV}$ (carbon) for which no enhancement is observed for other elements. An explanation for this is that the enrichment is accomplished by diffusion in the chromosphere, whereby collisions involving ionized particles are much faster than neutral-neutral collisions^{3,24}. In such a model, the element enrichment is governed not by the FIP proper but a related parameter, the ionization time in the chromosphere². Because Xe and Kr ionize relatively fast, as a result of their bulkiness, the observed Xe and Kr enrichments are expected²⁴, supporting the hypothesis that it is the time for ionization that underlies the FIP-effect.

In Fig. 2 we infer the secular variation of the heavy noble gas abundances in the corona by combining all single-grain data of this work with the on-line etch results on the very ancient lunar soil breccia 14301⁶. Error bars are rather conservative (see figure legend). The coronal abundances in the past 100 million years clearly differ from those in earlier epochs. Whereas the Kr/Ar ratio appears to increase steadily with time from about 1.4 times to 1.8 times the value in the Sun, the Xe/Ar ratio probably shows a

maximum around $\sim 1,000$ million years ago. (However, although a temporal variation is clearly confirmed, we also point out that the compositional bias in the heavy noble-gas abundances in the solar wind has not varied very much in the past several thousand millions years.)

Future experimental and theoretical work will be needed to clarify the causes of this variability. In high-speed solar wind emanating from polar coronal holes the FIP-effect is 2–3 times less pronounced than in the normal solar wind^{25,26}, a finding that has recently been extended to additional elements by use of the SWICS instrument on the Ulysses probe orbiting over the solar poles³. Thus, a higher relative contribution of high-speed solar wind in the ancient samples might explain their smaller Kr-Ar bias. However, the opposite temporal trends of Xe and Kr enhancement factors suggest that other processes are also involved. One explanation that has been discussed²⁷ is that the relative contribution of transient solar wind associated with coronal mass ejections has been higher in the past. Elemental abundances in this type of solar wind are very variable but not constrained well enough to rule on this proposal²⁸. A further observational constraint is that the Kr and Xe enhancement factors are basically the same for the solar wind and the higher-energy solar energetic particle component, as shown by the etch experiments⁶. Our results reinforce the unique importance of the lunar regolith for solar physics; not only does it enable us to analyse solar species that are too rare to be detected *in situ* with present-day instruments, but it also conserves a record of the ancient Sun not otherwise available. □

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A compound refractive lens for focusing high-energy X-rays

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THE development of techniques for focusing X-rays has occupied physicists for more than a century. Refractive lenses, which are used extensively in visible-light optics, are generally considered inappropriate for focusing X-rays, because refraction effects are extremely small and absorption is strong. This has led to the development of alternative approaches^{1,2} based on bent crystals and X-ray mirrors, Fresnel and Bragg–Fresnel zone plates, and capillary optics (Kumakhov lenses). Here we describe a simple procedure for fabricating refractive lenses that are effective for focusing of X-rays in the energy range 5–40 keV. The problems associated with absorption are minimized by fabricating the lenses from low-atomic-weight materials. Refraction of X-rays by one such lens is still extremely small, but a compound lens (consisting of tens or hundreds of individual lenses arranged in a linear array) can readily focus X-rays in one or two dimensions. We have fabricated a compound lens by drilling 30 closely spaced holes (each having a radius of 0.3 mm) in an aluminium block, and we demonstrate its effectiveness by focusing a 14-keV X-ray beam to a spot size of 8 μm .

The index of refraction for X-rays in matter can be written as $n = 1 - \delta + i\beta$, where β is the absorption index and δ is the

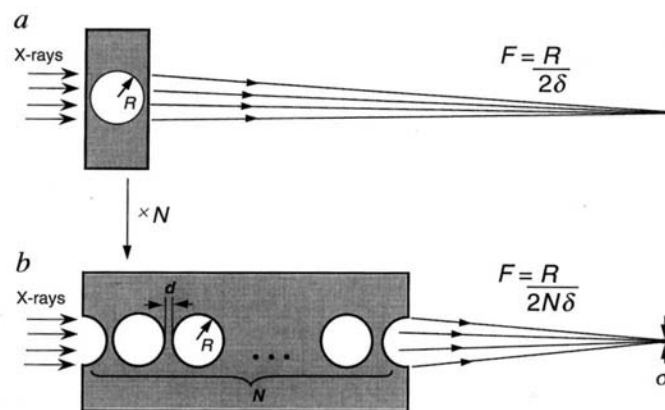


FIG. 1 Schematic diagram showing the principles of X-ray focusing by a compound refractive lens (CRL). As $(1 - \delta)$ is smaller than 1 (where δ is the decrement of the refractive index), a collecting lens for X-rays must have a concave shape. a, A simple concave lens fabricated as a cylindrical hole in the material. b, A CRL consisting of a number (N) of cylindrical holes placed close together in a row along the optical axis, focuses the X-rays at a distance that is N times shorter compared to a single lens. R is the radius of the holes, d is the spacing between the holes, λ is the X-ray wavelength, and F is the focal distance for a parallel input beam.

refractive index decrement. Refraction being very small (δ is typically between 10^{-5} and 10^{-7}), all attempts to date to build refractive lenses for X-rays have been unsuccessful. Recently, the discussion about refractive lenses has been revived. Suehiro, Miyaji and Hayashi³ have proposed a refractive lens of high-atomic-number (high- Z) material for focusing X-rays. Michette⁴

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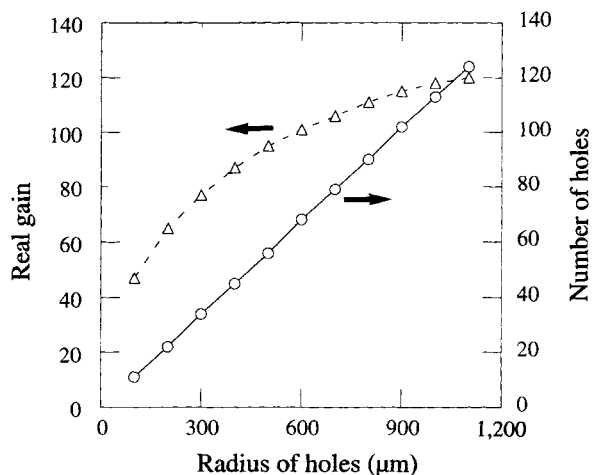


FIG. 2 Calculated number of holes N and real gain g versus radius of the holes R for a boron lens at fixed 1-m focal distance (10-keV X-rays). The number of holes increases linearly with increasing hole radius. Significant gain can be obtained with relatively few holes.

opposed the idea of using these lenses for soft X-rays because absorption dominates in that region. Yang⁵ considered the possibility of making X-ray refractive lenses for hard X-rays using low-Z materials. However, the low values of δ for low-Z materials implies lens radii of only a few micrometres. To overcome this problem, we propose the construction of a compound refractive lens (CRL), consisting of a linear array of many simple lenses manufactured in low-Z material (such as boron, carbon, aluminium, polymers or water). Figure 1 shows such an arrangement that results in a line focus of X-rays. A single lens (Fig. 1a) has a focal distance $F = R/2\delta$ where R is the radius of the lens. A compound lens with N holes has a focal length $F = R/2N\delta$. For example, for aluminium at an X-ray energy of 14 keV, $\delta = 2.8 \times 10^{-6}$. Hence $F = 54$ m for an individual lens, whereas the compound lens with, say, 30 holes brings the focal length into a range acceptable for many microfocus experiments ($F = 1.8$ m).

We made a theoretical analysis of the CRL based on the Fresnel–Kirchhoff approach. Here we present the main results demonstrating the useful properties of this type of lens, omitting the derivation of formulas.

First, the CRL acts as a normal conventional lens and we can apply the Gauss lens formula, which relates the source distance r_0 , the image distance r_i and the focal length F via $r_i = Fr_0/(r_0 - F)$. The diffraction-limited resolution of the lens σ_i is defined by an effective lens aperture A :

$$\sigma_i = \frac{\lambda r_i}{A} \quad (1)$$

where λ is the X-ray wavelength. For example, a lens of 500- μ m aperture with 1-m focal distance should have a resolution of 0.2 μ m.

To a first approximation, the aperture of the lens is $2R$. But if we neglect the absorption, only the central part of the cylindrical hole has the required parabolic shape of an ideal lens. Hence the aperture can be expressed as:

$$A_i = 2R \left(4 \frac{\lambda r_i}{R^2} \right)^{\frac{1}{4}} \quad (2)$$

But when absorption suppresses the contribution of the outer parts of the lens, the aperture is given by:

$$A_a = 2R \left(\frac{2}{\mu R N} \right)^{\frac{1}{2}} \quad (3)$$

Here $\mu = 4\pi\beta/\lambda$ is a linear absorption coefficient. The real

aperture of the CRL is the smaller of the two values given by equations (2) and (3).

An important feature of focusing optics is the intensity gain in the focal spot compared with the intensity which would have been obtained without a lens, using a collimated pinhole or slit. The ideal gain G , defined for a point source, is given by energy conservation as:

$$G = \frac{A}{\sigma_i} \left(1 + \frac{r_i}{r_0} \right) \quad (4)$$

When absorption defines the aperture, $G = 4\delta/\pi\beta$. The real gain g takes into account the source size σ_0 and X-ray absorption in the lens material:

$$g = aG \frac{\sigma_i}{\sigma_1} = a \frac{A}{\sigma_0} \left(\frac{r_0}{r_i} + 1 \right) \quad (5)$$

where $a = \exp(-\mu Nd)$ and $\sigma_1 = \sigma_0 r_i/r_0$ is the real focus size defined as a demagnified projection of the source size.

The real gain and the required number of holes are shown in Table 1 as a function of photon energy for boron and aluminium compound lenses. Figure 2 gives the number of holes in the array

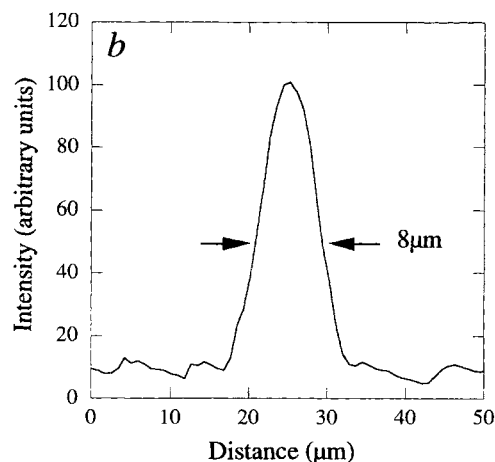
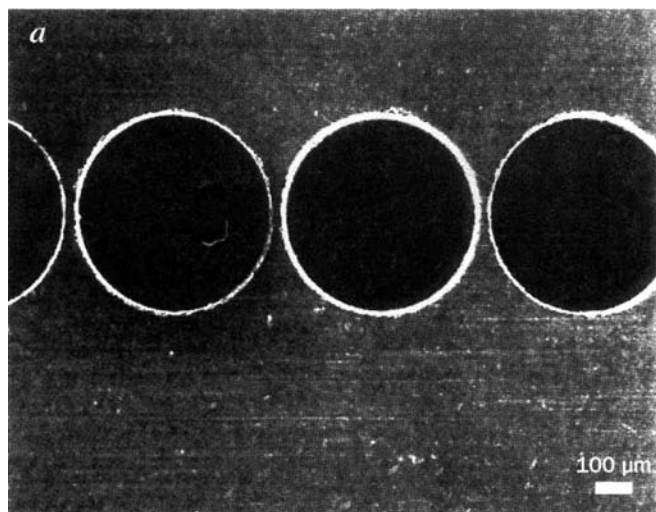


FIG. 3 a, Scanning electron microscope image of the tested compound refractive lens (CRL). 30 cylindrical holes of 300- μ m radius were produced in an Al–Cu alloy (with 4 wt% Cu) plate using a computer-controlled drilling machine. Spacing d , the minimum alloy thickness between the holes, was ~ 25 μ m. The length of the CRL was 19 mm. b, Measured profile of the focused X-ray beam taken at 1.8 m from the CRL. The width of the focal line is ~ 8 μ m, which almost corresponds to the source size of 150 μ m reduced by a demagnification factor $r_0/r_i = 20$.

TABLE 1 Calculated CRL parameters for boron and aluminium

Lens material	Energy (keV)	Number of holes	Effective aperture (μm)	Ideal gain	Real gain	CRL length (mm)
Boron	5	13	251	254	95	13
	10	55	211	359	93	55
	15	125	191	440	89	126
	20	222	177	508	84	224
	25	345	168	568	81	348
	30	501	160	622	78	506
	35	682	154	672	75	689
	40	892	149	719	73	901
Aluminium	5	11	85	22	0.2	11
	10	45	116	83	3	45
	20	184	163	336	18	186
	25	289	168	568	26	292
	30	417	160	622	31	421
	35	568	154	672	34	573
	40	743	149	718	37	750

Calculations were made under conditions typical for the ESRF beamlines: source size, 100 μm ; source-to-lens distance, 50 m. Lens parameters were as follows: spacing between holes $d = 10 \mu\text{m}$, hole radius $R = 500 \mu\text{m}$, focal distance $F = 1 \text{ m}$. The ideal gain (G) in the intensity at the focus for a point source was calculated according to ref. 4. The real gain (g) takes into account the finite source size and the attenuation of the X-rays owing to the absorption in the material between the holes⁵. The calculated focal spot size is $<1 \mu\text{m}$ in all cases.

and the real gain versus the radius R of holes in a boron CRL. The number of holes N increases linearly with R and quadratically with energy. The gain is mainly controlled by absorption. Acceptable hole radii are in the range 250–600 μm . This matches well the beam size of third-generation synchrotron radiation sources like the European Synchrotron Radiation Facility (ESRF). Low- Z material with a high density is the best choice as CRL material because δ is proportional $Z\rho$ whereas absorption varies as $Z^4\rho$, where ρ is the material density. Our estimates show that boron is a very promising lens material for all energies considered. However, aluminium is easier to machine with modern computer-controlled drilling, so it can be used as a reasonable alternative. The number of holes is limited by the reasonable lengths of the array and the requirement to make the distance d (Fig. 1) as small as possible.

We manufactured a CRL in an aluminium alloy; a scanning electron microscope image of the compound lens is given in Fig. 3a. The lens was tested at the optics beamline (D-5) at the ESRF. The 14-keV radiation from the bending magnet (radiation source size 150 μm) was selected by a Si-111 monochromator. The width of the focal line measured by pinhole scan (Fig. 3b) was 8 μm , with a gain of 3 over a similar size produced with slits, in good agreement with theoretical estimations.

Comparing the CRL with bent-mirror optics, we note that the CRL does not change the beam path, whereas the mirror does. The compound lens is more compact than a mirror, as no bending mechanism is needed. Requirements for surface quality are significantly lower than for mirrors owing to a transmission geometry of scattering. CRL alignment is straightforward. It is also reasonable to compare the CRL with a Kumakhov lens. At first glance, both lens types seem to have a similar approach; multiply a single element (a single capillary in a Kumakhov lens and a simple refractive lens in a CRL) to gain in optical performance. However, the Kumakhov lens is based on total reflection

in a bundle of capillaries whereas the CRL is based on refraction. Unlike the case of the CRL, the Gauss lens formula cannot be applied to the polycapillary optics; the Kumakhov lens works as a collimator to collect centimetre-wide neutron and X-ray beams, but with a modest resolution (100 μm). The CRL, having a smaller collecting area (up to millimetre), is well adapted to the beam properties of third-generation synchrotron radiation sources and is capable of micrometre and submicrometre resolution. Therefore the two lens types should be considered as complementary with their own fields of application.

The CRL described above is a cylindrical lens focusing only in one direction. Focusing in both directions, resulting in a point focus, can be achieved in two ways. The simplest is to use two arrays of cylindrical holes in a crossed geometry; in fact the quality of the focal spot may be improved by using three arrays of holes at 120° to each other in a rod with hexagonal shape. The more complex approach to point focusing uses thin-walled hollow plastic spheres 0.5 mm in diameter. When the spheres are aligned in a tube with 0.5 mm inner diameter, and when the space between the spheres is filled with a liquid, genuine two-dimensional focusing may be achieved. For instance, a water lens with a row of 300 spheres of 0.5 mm diameter has a focal lengths of 1.8 m for 30-keV X-rays.

A CRL is easy and cheap to make. It is possible to make a composite lens consisting of a set of parallel rows of holes with different focal distances or different hole radii. To change the focal distance or working-energy range, one can easily switch from one array to another simply by moving the composite lens. Parabolic-shaped holes can be produced in a CRL by applying new technologies⁶. Such holes would allow the length of a CRL to be reduced by a factor of five, while keeping the same performance. By choice of a proper material and geometry, it may be possible to use a CRL in neutron applications. □

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Mapping stress with ultrasound

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THE speed of sound in a solid is altered in the presence of mechanical stress. This effect can be exploited to form the basis of 'acoustic microscopy', whereby images of stress patterns in a material are obtained by monitoring the times of flight of sound pulses¹. The sensitivity of this approach is limited because wave speeds typically change by less than 1%, even when materials are stressed until they yield. Here we demonstrate a more sensitive form of acoustic microscopy. In an isotropic elastic medium, stress-induced anisotropies affect wave polarizations and phases, giving rise to interference between waves that would, in the absence of stress, remain in phase; the resulting patterns of interference between these waves reveal the underlying patterns of stress. This technique, by using acoustic waves of different wavelengths, permits the imaging of stress in objects ranging in size from microelectric devices to welds in pressure vessels.

The first example of the stress mapping technique makes use of the shear acoustic mode travelling back and forth across the specimen, generated by use of the microscope in an out-of-focus mode. In the absence of stress, the resultant shear waves would be polarized normal to their directions of propagation, in the vertical plane containing these directions—SV (shear-vertical) waves. Stress, however, splits an SV wave into two 'quasi-shear' waves, with their own wave speeds, and polarizations normal to each other and almost normal to their directions of propagation². Each produces two reflected quasi-shear waves (Fig. 1a). Allowance for two quasi-shear waves has been made in some applications²⁻⁴, but the effects of multiplication of shear arrivals with each reflection have not been considered. The vertical component of motion in any one of the waves couples with the fluid on return to the front face, to produce its own longitudinal wave, transmitted back to the transducer. These longitudinal waves return with phase differences that depend on the stress in the region sampled by the shear waves. The interference pattern that results provides a map of that stress.

The effect is illustrated by the following model calculation (Fig.

1a). The largest coupling between the incident pressure wave and the shear wave occurs at an angle of incidence that is well-defined in the absence of stress, and this is altered only slightly when stress is present. Let the two quasi-shear waves generated by such an incident wave have speeds v_1 and v_2 , and corresponding directions of propagation defined by angles α_1 and α_2 (related so that $\sin \alpha_1/v_1 = \sin \alpha_2/v_2$). The phase shifts that occur when the waves traverse the specimen are

$$\phi_1 = \frac{2\pi fd}{v_1 \cos \alpha_1}, \quad \phi_2 = \frac{2\pi fd}{v_2 \cos \alpha_2}, \quad (1)$$

where d is the thickness of the specimen. The wave speeds, as well as their polarizations, are easily calculated in terms of the stress, given the usual material parameters (density and Lamé moduli), together with the Murnaghan third-order elastic constants⁵. Each reflected wave has a corresponding shift when it returns to the top surface. There are thus arrivals associated with four distinct paths: one with shift $2\phi_1$, one with shift $2\phi_2$, and two with shift $\phi_1 + \phi_2$. The total vertical component of surface motion associated with these waves has the form

$$u(t) = \text{Re}\{\exp(2\pi if)\{A_1 \exp(2i\phi_1) + A_2 \exp(2i\phi_2) + (A_3 + A_4) \exp[i(\phi_1 + \phi_2)]\}\}. \quad (2)$$

Assume for illustration only, that $A_1 = A_2 = A_3 = A_4 = 1$. The amplitude of the wave defined by equation (2) is then easily shown to be

$$4 \cos^2 \left[\pi \left(\frac{fd}{v_1 \cos \alpha_1} - \frac{fd}{v_2 \cos \alpha_2} \right) \right] \quad (3)$$

Our second example of use of the microscope out of focus

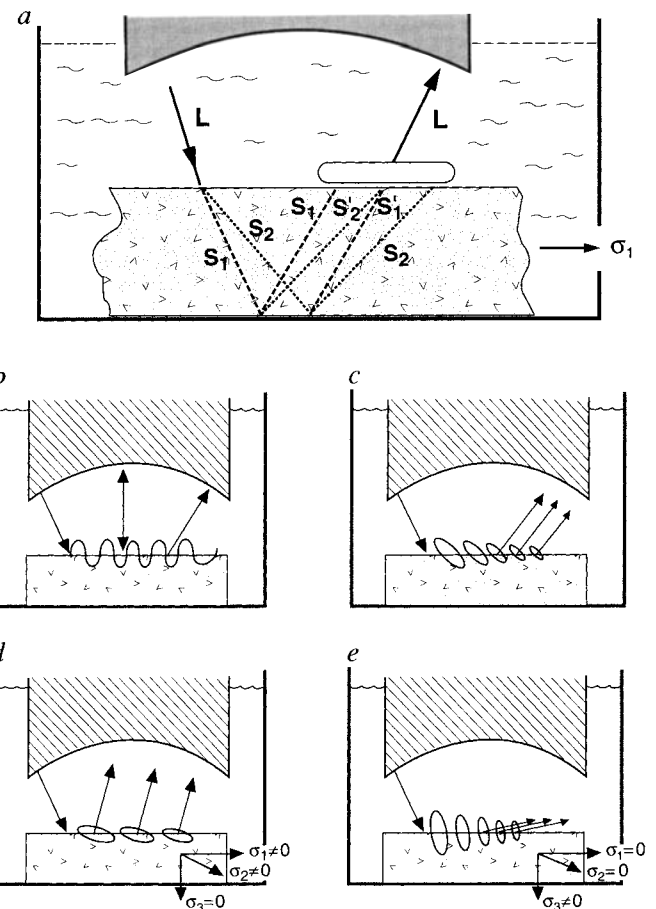


FIG. 1 a, The longitudinal angular arrival through water (marked L) generates in the stressed solid two quasi-shear waves travelling with different speeds. The longitudinal reflected and refracted waves are not shown. The two shear modes S_1 and S_2 , after reflection in the sample, create four reflected shear waves which interfere at the sample surface. The result of this interference will convert into a longitudinal wave in liquid and will be seen on the screen of the scope or computer as an acoustic pulse. An acoustic microscope gate placed at this pulse will receive the combined amplitude of the four shear waves, which depends upon the orientation and distribution of the shear stresses averaged over the sample thickness. b–e, The surface leaky wave created by defocused spherical transducer. The wave travels at the interface between water and sample (b). For isotropic elastic material, the image created by the surface leaky wave will be homogeneous. The characteristic asymptotic leakage angle⁶ is the direction of the main energy flow. The polarization plane of the wave is perpendicular to the Poynting vector (the vector of main energy flow) and is shown (c) as an ellipse of the particle displacement⁶. Hypothetically it can be predicted that the stresses σ_1 and σ_2 acting along the surface will cause direction-sensitive reorientation of the original polarization (d) so long as $\sigma_1 \neq \sigma_2$, and corresponding interference of signals received at the spherical lens. e, The postulated reorientation of the polarization plane when σ_3 has a gradient large enough for its influence to dominate. The darkest areas of the image will represent in this case the areas of highest stress. (Different wavelengths sample different depths; very short waves will show no effect because $\sigma_3 = 0$ at the surface.)

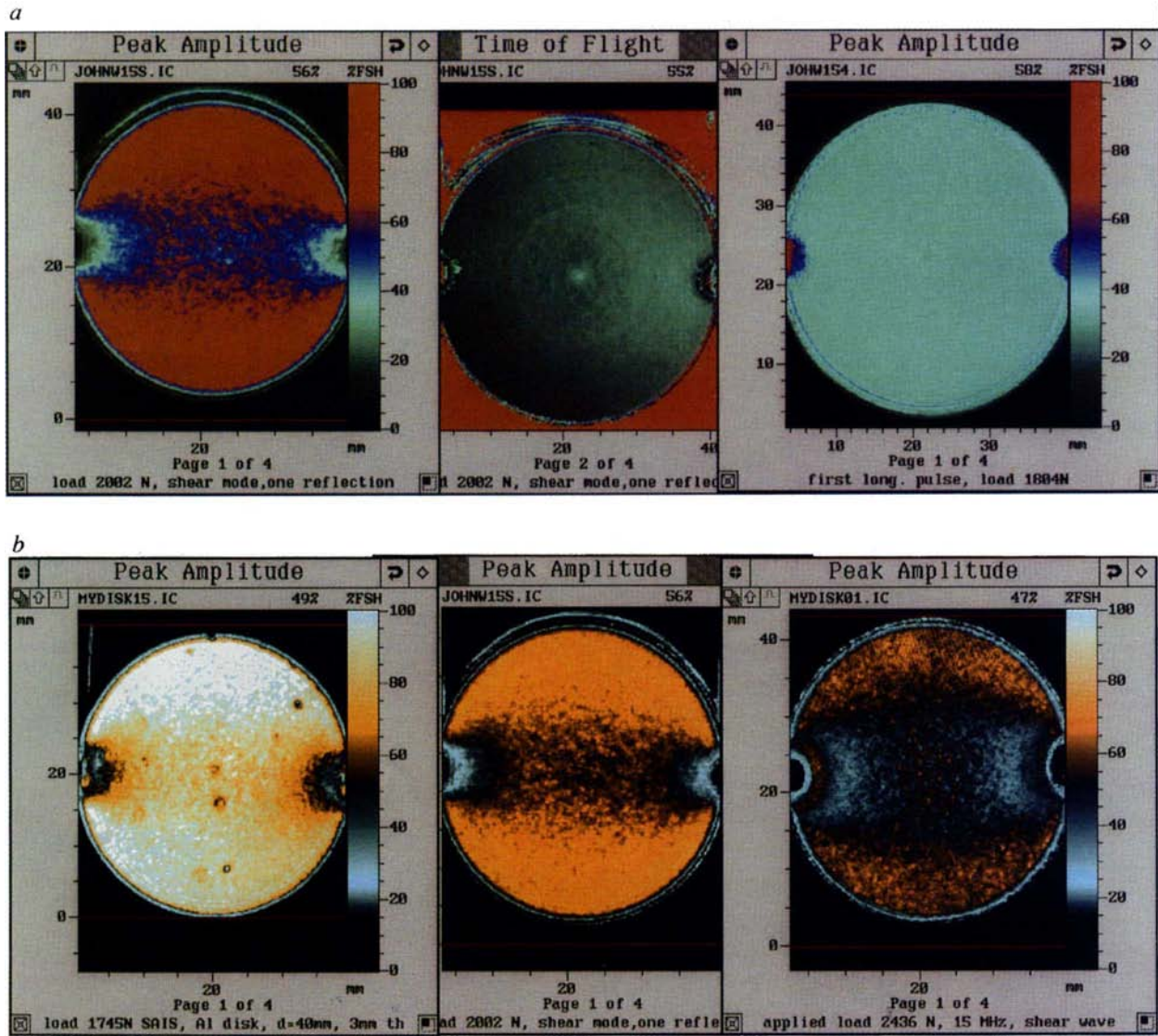


FIG. 2 *a*, Acoustic microscope images of a diametrically compressed (to 2,002 N) aluminium disk 3 mm thick and 40 mm in diameter. These images compare the sensitivity to stress of amplitude, time of flight and thickness scanning. A low-frequency scanning acoustic microscope (Sonix, Springfield, Virginia) with frequency range 1–150 MHz was used with 15-

MHz Panametrics V313, 15/25 transducer with $F = 12.7$ mm, PTF 193889. Images 1 and 2 were obtained under defocused conditions; image 3 was obtained with lens focused at the surface. *b*, Maps of stress at loads 1,745, 2,002 and 2,436 N, imaged by the shear pulse travelling twice through the sample thickness.

exploits its ability to generate 'leaky waves' propagating along the solid-fluid interface, and to receive the radiation produced by them (Fig. 1*b–e*). The precise angle of incidence for excitation of the leaky wave, which coincides with the angle at which radiation leaks from the wave, depends upon the wave speeds in the fluid and the solid⁶ and this influences the intensity and phase of the signal received by the microscope's transducer. Images of stress as represented by measured phase shifts of leaky waves were presented by Meeks *et al.*⁷. Their technique required the use of a special acoustic lens with anisotropic illumination pattern. This minimized interference between leaky waves traversing the sample in different directions (but would not eliminate it). In contrast, the present method actually relies on this interference, by direct monitoring of the resultant amplitude produced by leaky waves generated from use of a conventional spherical lens.

Figure 2 presents the shear-wave imaging of an aluminium disc subjected to diametral compression. Figure 2*a* compares the sensitivity of amplitude imaging (image 1) with the ultrasonic measurement of time of flight (image 2) at the same level of applied load, and also demonstrates that the stress produces no

noticeable change of thickness (image 3). Figure 2*b* presents shear-wave imaging at increasing load. The two split shear waves would be difficult to distinguish separately, because their travel time difference is so small for this sample, whose thickness is 3 mm. The amplitude that is recorded does depend sensitively on the level of stress.

Neglect of the multiple generation of quasi-shear waves upon reflection would lead to a signal with just two phase lags, $2\phi_1$ and $2\phi_2$ (for propagation twice across the specimen). This would give, in place of the values just discussed, $A_1 = A_2 = 1$, $A_3 = A_4 = 0$, yielding the usual interference formula

$$2 \cos \left[2\pi \left(\frac{fd}{v_1 \cos \alpha_1} - \frac{fd}{v_2 \cos \alpha_2} \right) \right]. \quad (4)$$

Use³ of the formula (4) instead of (3) generates the same patterns of contours, but for quantitative evaluation it is important to assign the correct values for the four amplitudes $A_1 - A_4$ and then for the amplitude of the combined wave that returns to the lens.

The recognition of multiple waves can also have implications

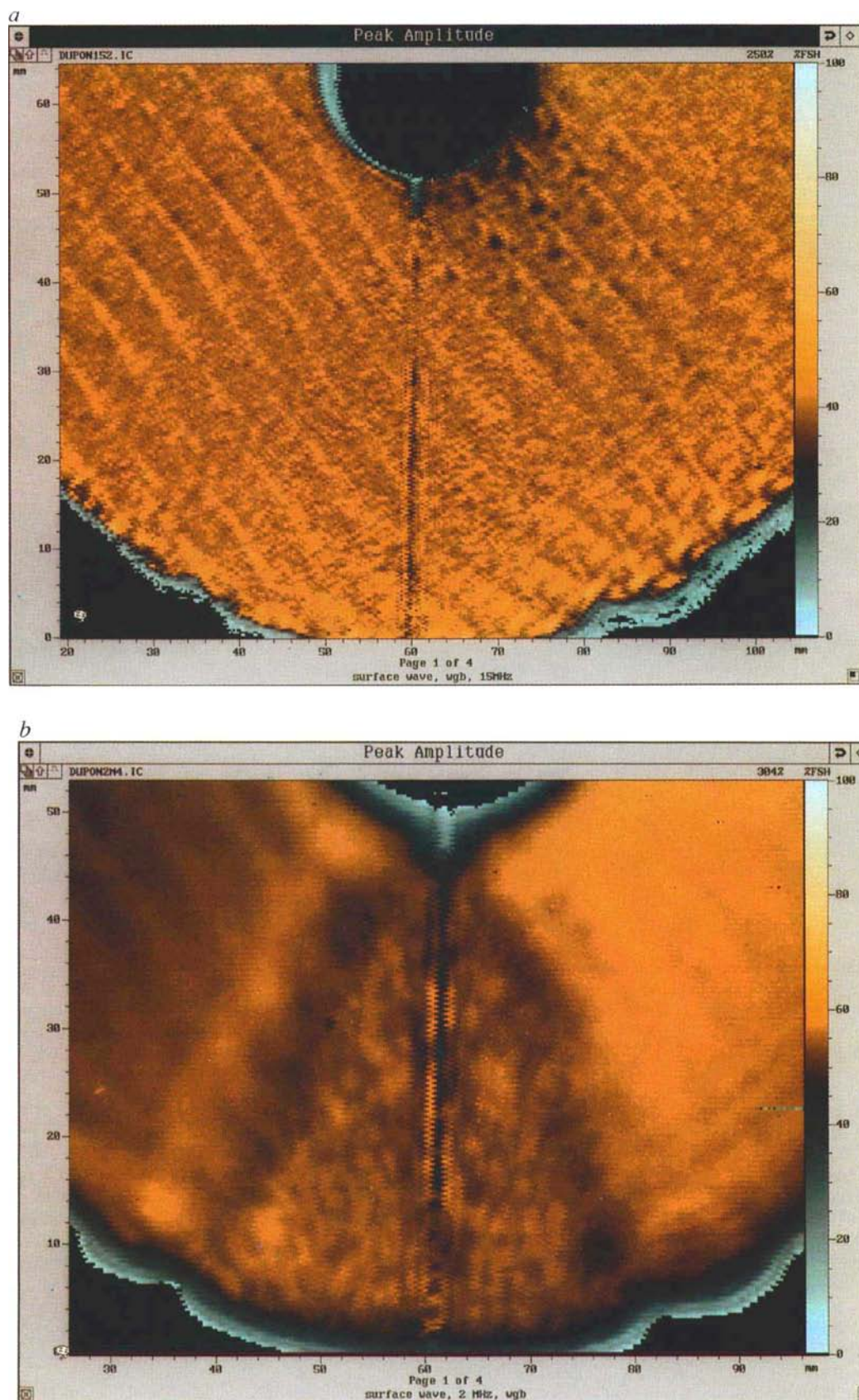
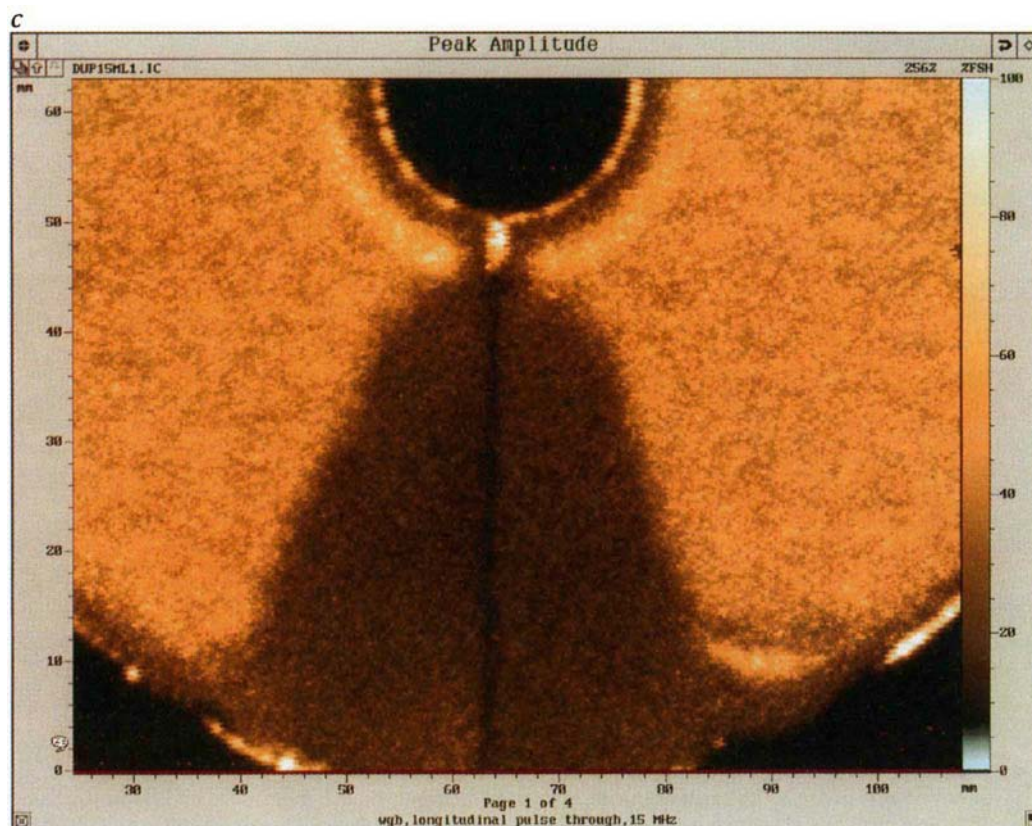


FIG. 3 Surface and longitudinal wave images of a 10-cm-thick section of a cylindrical Inconel pipe containing a weld. The first top image was obtained using surface waves at 15 MHz (a) and shows no appreciable variation due to stress. The image obtained by using the surface wave 7.5 times longer (2 MHz) shows the outline of the weld because it samples down to a depth where residual stress is present (b). c, Image obtained by scanning the

section of pipe using the longitudinal wave reflected once from the back of the specimen, in out-of-focus mode. Under the assumption that the axis of symmetry of the sample is a principal axis of stress, this provides an image of the sum of the two secondary principal stresses σ_1 , σ_2 averaged over the thickness of the sample.



for velocity measurements. For instance, attempts to record arrivals after multiple consecutive reflections have not achieved the anticipated improvement in accuracy⁴. This can be understood in terms of interference between increasing numbers of waves.

Figure 3 shows a section of a thick cylindrical pipe containing a weld, probed by leaky waves (Fig. 3a, b). Different frequencies produce differing amounts of penetration and permit control over the depth of material that is sampled. The top surface of the sample was ground. The intensity map obtained at 15 MHz (Fig. 3a) shows no appreciable variation, consistent with the absence of stress near the surface. Homogeneity between weld and parent material is also demonstrated. The corresponding map at 2 MHz (Fig. 3b) shows the outline of the weld, because it samples down to a depth where residual stress is present. Stress throughout the bulk of the weld is confirmed in Fig. 3c, which was obtained by transmission and reflection through the thickness of the specimen, of the longitudinal wave generated in out-of-focus mode.

Many other examples are available; further information is available on the World-Wide Web (<http://www.ctcms.nist.gov/~kfrankli/stresses.html>). Detailed theoretical analysis which we believe will enhance understanding and lead to improved technique for practical deployment is in progress. □

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Evidence for life on Earth before 3,800 million years ago

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It is unknown when life first appeared on Earth. The earliest known microfossils (~3,500 Myr before present) are structurally complex, and if it is assumed that the associated organisms required a long time to develop this degree of complexity, then the existence of life much earlier than this can be argued^{1,2}. But the known examples of crustal rocks older than ~3,500 Myr have experienced intense metamorphism, which would have obliterated any fragile microfossils contained therein. It is therefore necessary to search for geochemical evidence of past biotic activity that has been preserved within minerals that are resistant to metamorphism. Here we report ion-microprobe measurements of the carbon-isotope composition of carbonaceous inclusions within grains of apatite (basic calcium phosphate) from the oldest known sediment sequences—a ~3,800-Myr-old banded iron formation from the Isua supracrustal belt, West Greenland^{3,5}, and a similar formation from the nearby Akilia island that is possibly older than 3,850 Myr (ref. 3). The carbon in

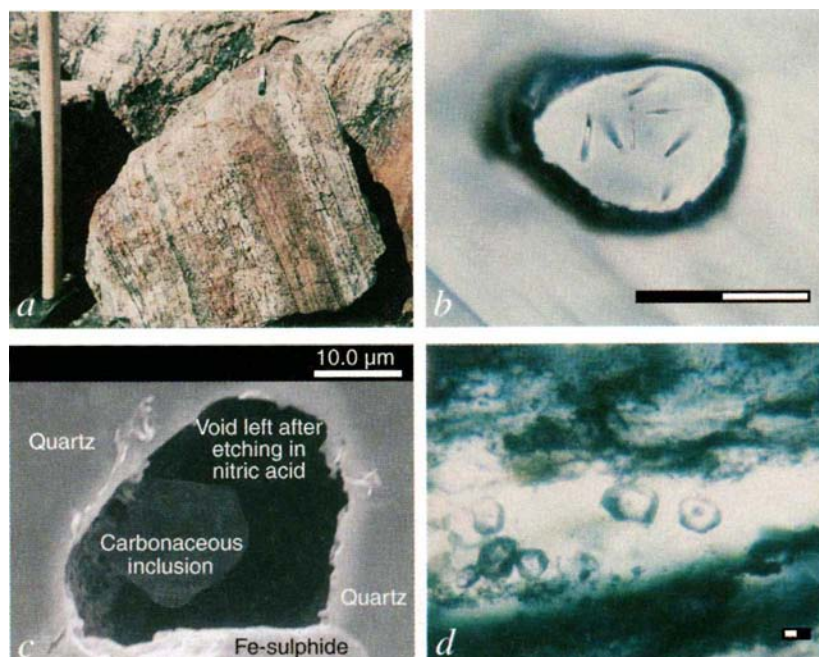


FIG. 1 a, Field exposure of the $\geq 3,850$ -yr-old Akilia island BIF³ (collected 150 km south of Isua, 63° 55' 40" N, 51° 41' 30" W; photograph by A. Nutman) in southern West Greenland. Finely alternating bands of magnetite (dark) and silicates (light) are evident on the broken-off boulder which is part of a ~ 5 -m-thick section of BIF (background) on the island; the rock hammer is ~ 40 cm tall. b–d, micrographs of anhedral, oblate apatite grains and associated carbon in early Archaean BIF. b, View of an apatite crystal in amphibole (grunerite), from the Akilia island BIF. The apatite was etched in 2% HNO₃ (120 s at room temperature) to uncover an envelope of opaque carbonaceous matter at the grain boundary; linear features in the crystal are fission tracks from the decay of intrinsic radionuclides revealed by the etching process (b and d are optical micrographs in transmitted light, oil immersion lens, plane polarized). c, Scanning electron micrograph of void left after treating apatite in 2% HNO₃ (1,800 s at room temperature), revealing an acid-resistant carbonaceous inclusion (centre) typical of those analysed by ion microprobe. d, Quartzitic microband from the Pilbara craton sediments, Western Australia, containing groupings of apatites with cores of organic matter along thin laminae of organic rich chert. Scale bars in b and d, 20 μ m; scale bar in c, 10 μ m.

the carbonaceous inclusions is isotopically light, indicative of biological activity; no known abiotic process can explain the data. Unless some unknown abiotic process exists which is able both to create such isotopically light carbon and then selectively incorporate it into apatite grains, our results provide evidence for the emergence of life on Earth by at least 3,800 Myr before present.

Because of the unique chemical properties of phosphate and its fundamental role in a large variety of biochemical processes⁴, the participation of phosphorus in biogeochemical cycles must be a primitive phenomenon. Authigenic phosphate minerals are a significant sedimentary component of sediments^{5–8}. The principal constituent of modern authigenic phosphate minerals in marine sediments is carbonate (hydroxy)fluorapatite (CHFA), $\text{Ca}_{10}(\text{PO}_4)_6-x(\text{CO}_3)_x(\text{F}, \text{OH})_{2+x}$. In the marine environment, organisms participate in concentrating organic phosphate from solution and by recycling organic phosphate species from decaying P-rich bioorganic matter^{7–11}. Microorganisms are well known to segregate calcium from magnesium, and actively nucleate CHFA by means of specific oligopeptides^{8–10}. Due to this common formation of authigenic CHFA, microcrystalline aggregates of apatite in modern as well as ancient sediments characteristically are intergrown with organic matter^{6–11}. During diagenesis the aggregates recrystallize, eventually forming single apatite crystals with inclusions of carbonaceous material, which after extensive metamorphism crystallizes to graphite.

Organisms are capable of depositing apatite outside thermodynamic equilibrium in sea water with $\text{pH} < 8.5$ and $[\text{Mg}]:[\text{Ca}] > 0.1$ (refs 10–12), which indicates the potential value of phosphate microminerals and their associated carbonaceous inclusions as indicators of biological activity in ancient sedimentary chemical precipitates, such as chert and banded iron formations (BIFs)¹¹. The potential biogenic significance of the apatite alone can only be realized if the range of Mg/Ca ratios and the pH in the source solution can be estimated independently. Even in exceptional cases, BIFs have at most minor fractions of clastic apatite derived from the weathering of igneous rocks^{13,14}. However, such igneous apatite is free of carbon inclusions and is resistant to dissolution in natural waters; it therefore has minimal interaction with marine biogeochemical cycles and, moreover, is a relatively minor mineral constituent in most igneous rocks and their weathering detritus. But metasedimentary apatite from early Archaean BIFs, if found to contain isotopically light carbonaceous

inclusions diagnostic of a bioorganic origin, might be one of the few distinguishable traces of early life in the Earth's sediments.

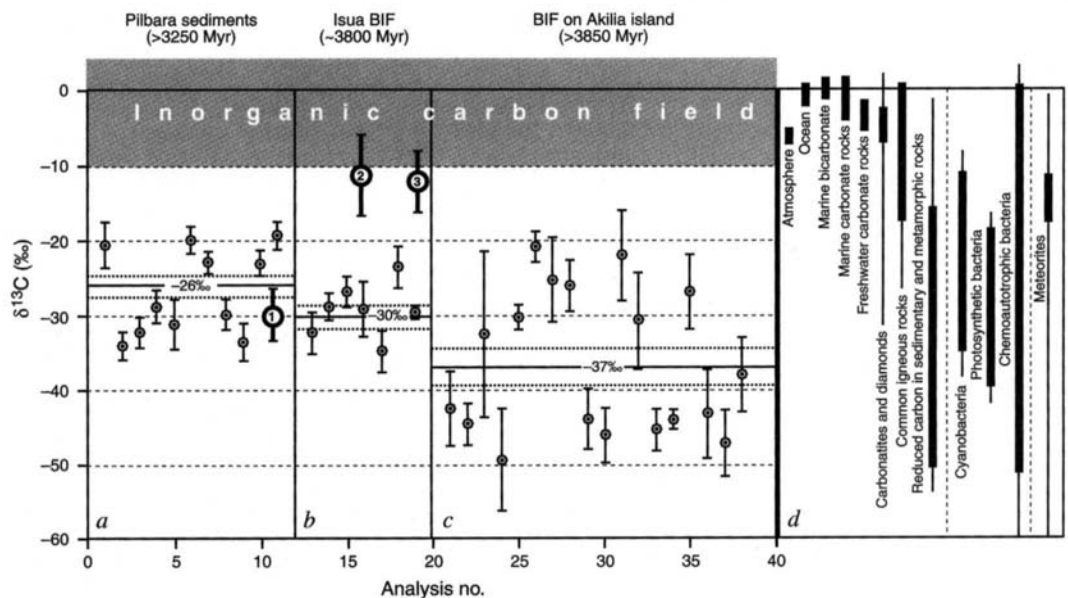
Carbon isotopic measurements of carbonaceous matter in sedimentary rocks have provided insights into bioorganic pathways and the evolution of early life with or without the presence of identifiable microfossils^{1,2,15–18}. However, conventional methods of mass spectrometry lack the sensitivity to analyse carbon isotopes in individual apatite inclusions which are typically $\sim 10 \mu\text{m}^3$ and contain ~ 20 pg carbon. The ion microprobe permits the study of isotopic variations at the scale of 10–20 μm spots²⁰. To enhance the accuracy of measurements, sputtering of each inclusion was generally continued until a large fraction of the target had been consumed. The required high sensitivity must be maintained at the relatively high mass resolving power ($M/\Delta M \approx 4,000$) necessary to separate interfering $^{12}\text{CH}^-$ ions from $^{13}\text{C}^-$. We have tested the hypothesis that carbonaceous inclusions contained in apatite from early Archaean sediments are biogenic by using an ion microprobe to perform *in situ* carbon isotope measurements of such mineral microdomains in cherts and BIFs from Western Australia ($\geq 3,250$ Myr) and from West Greenland ($\geq 3,700$ Myr).

The Pilbara craton of northwestern Western Australia contains well preserved volcano-sedimentary sequences with ages between $\sim 3,000$ and 3,500 Myr (refs 20, 21). Within the Warrawoona Group, cherts from the Apex Basalt ($3,450 \pm 16$ Myr)²² contain the oldest microfossils yet identified, some of which resemble extant chemoautotrophic and photoautotrophic prokaryotic morphotypes^{1,2}. Whole-rock carbon isotope ratios of kerogen in the Warrawoona sediments have been interpreted to infer that photosynthesizing, or even cyanobacterium-like, organisms were already active by 3,500 Myr (refs 1, 2, 16–19). The apatite intergrowths with organic matter we report here are from lower greenschist facies chert of the ($> 3,250$ Myr) Nickol Well unit of the Roebourne belt, west Pilbara Archaean succession²² (R. Buick, personal communication).

The Isua supracrustal belt in West Greenland contains large volumes of early Archaean BIF and meta-chert^{14,23–25,35}. As BIFs are of sedimentary origin, these rocks are at least as old as their metamorphic age of 3,700 Myr^{3,23–26,35}. Isua rocks used in this study have been metamorphosed to amphibolite facies; details of the mineralogy and petrographic relationships of the Isua rocks have been given elsewhere^{23,24,35}.

An early Archaean BIF encompassed within a layered amphi-

FIG. 2 Isotope compositions of carbonaceous inclusions in individual apatite grains from early Archaean sediments measured by ion microprobe. a, West Pilbara sediments, Roebourne Belt, Western Australia ($>3,250$ Myr), samples courtesy of K. Sugitani; the data indicated by '1' are previous whole-rock measurements¹⁵⁻¹⁷ of Warrawoona Group sediments for comparison; b, Isua supracrustal belt BIF ($>3,700$ Myr; field sample no. 3381, Isukasia, West Greenland; courtesy of E.I. Robbins and P.W.U. Appel); '2' and '3' indicate respectively previously whole-rock measurements reported by Schidlowski *et al.*¹⁸ and Hayes¹⁹. c, BIF from Akilia island ($\geq 3,850$ Myr) in southern West Greenland³. d, Carbon isotope variations found in nature. Standard deviations for the ion microprobe data are indicated by the vertical lines (1σ). Dotted lines above and below the weighted means of the data correspond to the 2σ confidence interval.



METHODS. Cleaned rock chips, taken several centimetres away from weathering surfaces and free from cracks, were cored to yield 25-mm-diameter rock disks of 5 mm thickness. These were polished with alumina powder in distilled water and drilled with an ultrasonic microcorer to produce a 3-mm-diameter hole at their centres, then sonically cleaned in successive ethanol and ultrapure water baths before being dried in air. Plugs 3 mm in diameter of pelletized USGS 24 graphite standard ($\delta^{13}\text{C}_{\text{PDB}} = -16.0\text{‰}$) were inserted into the central hole and the section was then Au-coated. Carbonaceous inclusions in the apatite were sputtered by a focused Cs^+ beam in the CAMECA ims1270 ion microprobe at UCLA, and charge compensation during negative ion extraction was maintained by using a normal-incidence electron gun. Carbon isotopic measurements were per-

formed by standard ion microprobe techniques^{19,32} utilizing magnetic peak switching at high mass-resolving power and ion counting with an electron multiplier. The carbon isotope ratios, corrected for deadtime and instrumental mass fractionation, are reported relative to the VPDB standard using the conventional delta notation. The mass-fractionation correction procedure assumes that the degree of bias for the lighter isotope inherent in the sputtering process is the same for the carbonaceous inclusions in the apatite as for the graphite standard. Measurements on a suite of kerogen samples differing by a factor of ~ 7 in H/C ratio show that the effect of such structural and compositional variations between the standard and the sample is small ($<2\text{‰}$) in agreement with earlier, less precise findings³³. The inorganic carbon field is the region of carbon isotopic compositions defined as characteristic of inorganic carbonate carbon and non-bioorganic reduced carbon.

bolite and ultramafic complex on Akilia island, southern West Greenland³⁴, is cut by a deformed quartz-dioritic sheet dated at $3,860 \pm 10$ Myr (ref. 3), providing a possible minimum age for the transected sedimentary unit. The sample used in this study (G91-26) comes from a well-preserved layer of BIF consisting of quartz (30%), clinopyroxene (25%), orthopyroxene (20%), amphibole (15%), magnetite (5%), iron sulphides ($\sim 1\%$) and other minerals ($<1\%$) including apatite, but no observable carbonate. The unit meets the criteria of James (ref. 26) for a silicate facies to low-grade oxide facies BIF. Anhedral oblate to lozenge-shaped apatite grains, occurring either individually or in groups, and resembling those found in the younger Pilbara cherts and Isua supracrustal belt samples cited above, are typically $10\text{--}15\text{ }\mu\text{m}$ in diameter and $30\text{--}40\text{ }\mu\text{m}$ in length (Fig. 1) and frequently contain inclusions and envelopes of graphitized carbon. Apatite grains occur in the pyroxenes, quartz, amphibole and (rarely) in magnetite, and when found in groups, are present as trains parallel to banding. In contrast, $\sim 3,860\text{--}3,870$ Myr orthogneisses from the same locality³, and transecting and encompassing the supracrustals, contain igneous apatites that are devoid of graphite inclusions, are compositionally distinct, and are associated with common igneous phases such as feldspar not found in chemically precipitated sediments like BIF.

The stable isotopes of carbon are partitioned as a result of both equilibrium exchange reactions and kinetic effects, which are due to metabolic mechanisms as well as inorganic processes such as evaporation, diffusion and condensation. Kinetic isotopic fractionation between organic and inorganic carbon results in marked enrichment of the light isotope in the bioorganic component by several per cent (refs 16, 17). Hence, bioorganic materials,

including carbonaceous fossils, are typically characterized¹⁵⁻¹⁸ by $\delta^{13}\text{C}$ values of -20 to -35‰ in the case of most photoautotrophic bacteria, and can be¹⁶⁻¹⁸ as light as -50 or -60‰ for products of microbial communities apparently involved in the recycling of methane. In contrast, inorganic carbon is usually heavier than about -10‰ , with a typical range between $+5$ to -5‰ (refs 16, 17).

In situ ion-microprobe measurements of occluded carbon in apatite micrograins from the Akilia island BIF yield a range of $\delta^{13}\text{C}$ values from $-21(\pm 2)\text{‰}$ to $-49(\pm 7)\text{‰}$, with a weighted mean of $-37(\pm 3)\text{‰}$ (Fig. 2). Because of the micrometre size of the irregular samples embedded in apatite, the precision and accuracy of individual measurements are typically $\pm 5\text{‰}$ (1σ) and encompass counting statistics plus an extra component for fluctuation of count-rates during analysis. Isotope results for carbonaceous inclusions in the Pilbara sediments and Isua BIF yield weighted means of $-26(\pm 3)\text{‰}$ and $-30(\pm 3)\text{‰}$, respectively. The results for the Pilbara sediments agree with previous whole-rock values obtained by conventional mass-spectrometric techniques for Warrawoona Group sediments¹⁶⁻¹⁸. All measured values from our early Archaean apatite inclusions are well resolved from what are generally considered to be inorganic carbon values.

To evaluate the presence of life in the previously oldest known sedimentary rocks, carbon isotope ratios were measured in acid insoluble carbonaceous residues (kerogens) of bulk samples from the $\geq 3,700$ -Myr Isua supracrustal belt BIF¹⁸. These measurements yielded mean $\delta^{13}\text{C}$ values of -11 to $-15(\pm 5)\text{‰}$ that have been interpreted as indicating photoautotrophic carbon fixation. However, these previous values are close to the range of inorganic carbon $\delta^{13}\text{C}$ ratios, possibly owing to isotopic exchange with

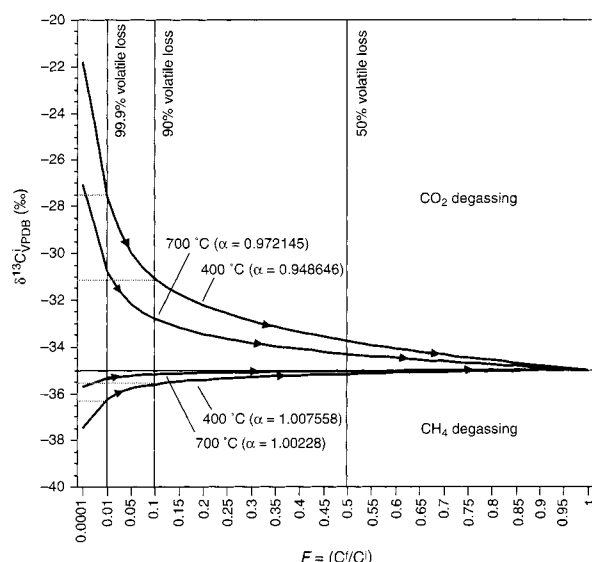


FIG. 3 Theoretical changes in the $\delta^{13}\text{C}$ values of organic carbon by degassing of CO_2 and CH_4 using a Rayleigh distillation process (open-system behaviour)³⁴ $\delta^{13}\text{C}^{\text{f}} = [\delta^{13}\text{C}^{\text{i}}/F^{(\alpha-1)}]$ which assumes a continuously reactive (graphitic) residue and immediate loss of gas from the system with lack of continuous isotopic equilibrium between the evolved gas and the residue. The F -values are the mole ratios of the final residuum (C^{f} ; carbon inclusions) to the initial carbon contents of the apatites (C^{i}), where $\delta^{13}\text{C}^{\text{f}}$ is

the final value of the organic carbon inclusions as measured *in situ* in the apatites. The α -values ($\alpha = K_{\text{B}}/K_{\text{A}}$, which is the ratio of the equilibrium constants of isotopic exchange reactions at a given temperature) for the equilibrium isotopic fractionation factors between the evolved species CH_4 (graphite-methane; $\text{C}-\text{CH}_4$), CO_2 ($\text{C}-\text{CO}_2$) versus graphite (C) at temperatures between 400 and 700 °C, the thermal metamorphic conditions experienced by the Akilia island BIF³⁴, are based on thermodynamical calculations^{33,36,37}. Depending on the oxygen fugacity (f_{O_2}) of the system, metamorphism can result in the release of different proportions of CO_2 and CH_4 . In both the CO_2 and CH_4 degassing, the isotopic ratio of the inclusion ('residue') would tend to increase or decrease depending on whether the expelled fluid preferentially partitions the light isotope (CH_4) or the heavy isotope (CO_2). The $[\text{CO}_2]/[\text{CH}_4]$ ratio of evolved fluids from graphite tends to increase with increasing temperature and/or decreasing pressure because of the reaction $2\text{C} + 2\text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{CH}_4$. Under high f_{O_2} conditions and at high temperatures, graphite reacting to form CO_2 could yield in the thermodynamical model an isotopically lighter residue during progressive metamorphism; the graphite content in the apatite would tend in all of these cases to continually decrease. Assigning $\delta^{13}\text{C}^{\text{i}} = -35\text{‰}$, the figure demonstrates that even under these hypothetical and extreme conditions resulting in the loss of CO_2 of 99.9% ($F = 0.001$) of the original carbon contained in the apatites, derivation of $\delta^{13}\text{C}^{\text{f}}$ from the inorganic isotopic field ($+5\text{‰} \geq \delta^{13}\text{C}^{\text{f}} \geq -10\text{‰}$) cannot be achieved, rather the range obtained is $-27.5\text{‰} \geq \delta^{13}\text{C}^{\text{f}} \geq -36.5\text{‰}$. A theoretical F -value of 2.54×10^{-11} is required to go from $\delta^{13}\text{C}^{\text{i}} = -10\text{‰}$ (the lowermost bounds of the inorganic field in Fig. 2) to $\delta^{13}\text{C}^{\text{f}} = -35\text{‰}$. These calculations provide extremes in both starting compositions and open-system behaviour of the carbonaceous inclusions during metamorphism. A kinetic model would give results that only increase the $^{13}\text{C}/^{12}\text{C}$ ratio, and lead to $\delta^{13}\text{C}^{\text{f}}$ values $\geq \delta^{13}\text{C}^{\text{i}}$. The x -axis reflects a scale change in F from linear ($F = 1.0-0.1$) to nonlinear ($F = 0.1-0.0001$) as a means of highlighting changes in $\delta^{13}\text{C}^{\text{f}}$ under extreme conditions of conversion of graphite to CO_2 or CH_4 .

carbonate carbon present in the Isua rocks during metamorphism, so they have been regarded as ambiguous¹. Some carbon in igneous rocks is observed to have intermediate isotope ratios in the range of -10 to -20‰ , but these could reflect bioorganic contamination from assimilated sediments²⁷. Miller-Urey spark-discharge laboratory experiments, carried out to simulate hypothetical hydrogen-rich primitive Earth atmospheres, yield organic bulk reaction products which are²⁸ isotopically heavier than -10‰ and that could not have contributed to the carbon in the apatites. We can rule out reduced carbon from carbonaceous meteorites (the richest contain ~ 3 mass% reduced C), as carbon isotope ratios for these generally cluster at²⁹ -11 to -18‰ and there is no reason to expect meteoritic carbon to be selectively associated with apatite in BIF. Regardless of the modes of origin for the carbon components in the various materials mentioned above, the $\delta^{13}\text{C}$ values for the carbon inclusions in apatite are 10 – 15‰ lighter still than the $\delta^{13}\text{C}$ values seen in such abiogenic samples, and are characteristic of the range of carbon isotopic compositions for bioorganic matter (Fig. 2).

For strongly negative carbon isotope values in metamorphosed sediments to be convincingly interpreted as unaltered products of bioorganic fractionation, it is necessary to analyse the magnitude and sign of such effects that could have perturbed an original distribution. Empirical studies have shown that the loss process of CO_2 from the oxidation of organic matter is kinetically controlled, and the evolved CO_2 is isotopically lighter than the source organic carbon. Hence, loss of volatiles from thermally degrading organic matter leads to the residual organic matter being isotopically enriched in ^{13}C . However, to investigate theoretical scenarios where progressive thermal metamorphism in principle could lead to enrichment of residual carbon in ^{12}C , we evaluated possible changes in the $\delta^{13}\text{C}$ value of organic matter included in apatite under both thermodynamically open-system (Rayleigh) and closed-system (single-step) behaviours. Depending on the oxygen fugacity of the system, metamorphism can result in the release from carbonaceous matter of different proportions of CO_2 and CH_4 fluids, but a loss of methane which partitions the light isotope can never produce isotopic compositions lighter than the starting materials. On the other hand, the escape (during dia-

genesis and metamorphism) of isotopically heavy CO_2 evolved from the organic matter trapped within the apatites could, in such a theoretical model, drive the residue to lighter isotopic values. The most extreme isotopic shifts would result from a Rayleigh distillation process, which assumes a continuously reactive (graphitic) residue and immediate removal of CO_2 from the system. The progressive change in $^{13}\text{C}/^{12}\text{C}$ of the graphite residue by such a Rayleigh-type process is shown in Fig. 3 as a function of the fraction of carbonaceous matter consumed in degassing to either CO_2 or CH_4 in the apatite. This figure shows that, for any degree of degassing and without consuming all of the carbon inclusion in the process, it is not possible to generate a final value (C^{f}) of $\delta^{13}\text{C}^{\text{f}} = -35\text{‰}$ from an inorganic initial material (C^{i}) with $\delta^{13}\text{C}^{\text{i}} > -10\text{‰}$. In fact the thermodynamically determined Rayleigh evolution lines do not intersect the inorganic carbon field except toward the limit of $F = 1/\infty$, where F is the mole fraction of remaining carbon. We assert that neither by kinetic nor by thermodynamic arguments, can loss of volatiles by thermal degradation of organic matter modify isotopically heavy abiogenic reduced carbon to make it resemble biogenic organic carbon.

In the CHFA mineral structure, carbonate primarily substitutes for $[\text{PO}_4^{3-}]$, and less frequently for $[\text{OH}^-]$. Thermally induced decarbonation of CHFA occurs between 400 and 800 °C via the decarboxylation of structural CO_3^{2-} , and possibly of $[\text{CO}_3^{2-}, \text{OH}^-]$ and $[\text{CO}_3^{2-}, \text{F}^-]$ ion pairs³⁰ leading to the formation of stable fluorapatite. However, there is no known mechanism that can reduce structural CO_3^{2-} in CHFA, or its decarbonation product CO_2 , at low partial pressure of hydrogen, to produce carbonaceous inclusions in apatite. Any such hypothetical carbon formed by reduction of carbonate or the evolved CO_2 would remain within the inorganic field of carbon isotope ratios anyway. Moreover, a simple mass-balance calculation shows that even 100% efficiency of such an assumed carbonate-reduction process could not supply the observed volume of carbonaceous inclusion contained in each of the apatites. The association of apatite with carbon is observed in the Pilbara sediments as well as in other younger, unaltered modern sediments, and in laboratory cultures of microorganisms; these features cannot be explained by metamorphism. We therefore conclude that metamorphic effect

are not responsible for the association of isotopically light carbonaceous inclusions in metasedimentary apatite.

Together with the intergrowth of carbonaceous matter with apatite in BIF from Akilia island, we conclude that the isotopic results reported here give strong evidence for life on Earth by 3,850 Myr. Although this finding pushes back the horizon for the emergence of life by 300–400 million years, it is not entirely unexpected¹, given also the apparently evolved nature of lifeforms

at ~3,500 Myr. However, the 'late heavy bombardment' (>3,800 Myr), documented in the lunar record, has been speculated to place an upper limit on the age of a continuous terrestrial biosphere³¹. The evidence for life presented here overlaps this critical time period and shows that if the accretion models are realistic, such a bombardment did not lead either to the extinction of life or the perturbation of the finely laminated >3,850-Myr BIF preserved on Akilia island. □

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Activation of floral meristem identity genes in *Arabidopsis*

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THE *Arabidopsis* floral meristem-identity genes *APETALAI* (*API*) and *LEAFY* (*LFY*) confer floral identity on developing floral primordia^{1–4}, whereas *TERMINAL FLOWER* (*TFL*) is required to repress their expression within shoot and inflorescence meristems^{1,5}. *LFY* and *API* are expressed in floral primordia in response to environmental conditions, such as day length, which regulate the onset of flowering, and presumably also in response to the action of genes that influence flowering time. However, the relationship between these flowering-time genes and the floral meristem-identity genes has been difficult to assess because flowering time is determined by several interacting genetic pathways^{6,7}. Here we describe a method to regulate expression of the flowering-time gene *CONSTANS* (*CO*) and demonstrate that *CO* expression is sufficient to trigger flowering, irrespective of day length. In response to *CO* expression, transcription of *LFY* and *TFL* is initiated rapidly, whereas transcription of *API* occurs much later. We propose that *CO* acts within a genetic pathway that is sufficient to activate *LFY* and *TFL* transcription, but that rapid activation of *API* requires an additional pathway.

Flowering of *Arabidopsis* occurs rapidly under long days (LDs) containing 16 hours light, and is delayed under short days (SDs) of 10 hours light (Fig. 1). Over 20 mutations that delay flowering under LDs have been described. The genes affected in these

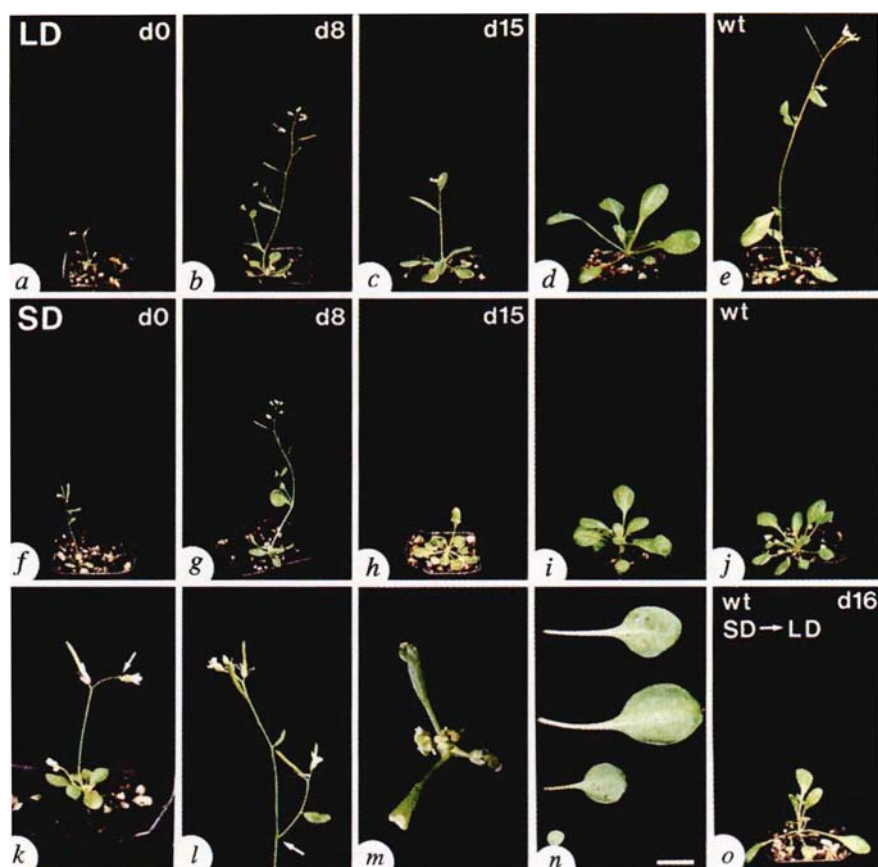
mutants have been placed in at least three pathways based upon double-mutant phenotypes and the response of the mutants to environmental conditions^{6,7}. One of the pathways is required to promote flowering in response to daylength, and includes the *CO* gene. Mutations in *CO* delay flowering under LDs, but have no effect under SDs^{8–10}, suggesting that *CO* promotes flowering in response to LDs. Flowering time is likely to be mediated, at least in part, by transcriptional regulation of *CO*, because *CO* messenger RNA is more abundant under LDs than SDs¹⁰. Furthermore, the *CO* mRNA is rare, and reducing *CO* gene dosage in heterozygous plants⁹ or increasing *CO* dosage in transgenic plants¹⁰ delays or promotes flowering respectively.

To examine the effect of *CO* expression on floral meristem-identity gene transcription and on the regulation of flowering by day length, we constructed a transgene designed to express the *CO* mRNA at high levels independently of day length, and to permit the activation of *CO* function at different stages of development. A transcriptional fusion between the *CO* complementary DNA and a strong viral promoter (cauliflower mosaic virus (CaMV) 35S) was used to increase expression of the mRNA. Furthermore, in order to regulate activity of the *CO* protein, the translational stop codon of *CO* was removed and replaced with a 287-amino-acid segment of the rat glucocorticoid receptor. This segment can inactivate plant transcription factors in the absence of the steroid ligand, but activity of the protein is restored in the presence of the ligand dexamethasone^{11,12}. Transgenic *co-2* mutant plants containing the gene fusion between *CO* and the glucocorticoid receptor (*CO:GR*) were constructed.

To assess the effect of *CO:GR* on flowering time, the response to applications of dexamethasone at different stages of development was measured in detail for plants grown under both LDs and SDs (Figs 1 and 2). Dexamethasone was supplied through the soil to the roots of the plants at various times from day 0 until 26 days after sowing (see Methods). This treatment had no effect on the flowering time or morphology of wild-type plants, nor of *co-2*

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FIG. 1 Flowering of *Arabidopsis* plants carrying *CO:GR* in response to dexamethasone. All plants are 31 days old and were grown under long days (a–e) or short days (f–j). Dexamethasone was added at different times after sowing; day 0 (d0; a, e), d8 (b, g), d15 (c, h). Untreated transgenic plants flowered late under long days because of the presence of the *co-2* mutation (d), or because flowering of *Arabidopsis* is delayed under short days (i). Wild-type *Landsberg erecta* was also grown under long and short days (e, j). k, Plant in a, at high magnification; arrow indicates a small terminal flower that failed to develop. l, Secondary inflorescences on dexamethasone-induced plants are often formed without a subtending cauline leaf (arrow). m, Top view of an induced plant, showing the carpelloid structure that often terminates development of the shoot. n, The third leaf of plants grown under long days; from top to bottom *Landsberg erecta*, and plants induced with dexamethasone at d26, d15 or d0. o, A wild-type plant grown under short days and transferred to long days on d16.



mutants. However, *CO:GR* plants flowered earlier when treated with dexamethasone. When dexamethasone was applied before day 20, LD-grown *CO:GR* plants formed fewer leaves than untreated controls (Fig. 2); *CO* can therefore act to promote flowering at any stage in plant development from early in germination until the time when flowers are formed by *co* mutants. Plants growing under LDs produced the same number of leaves as those growing under SDs when they were treated with dexamethasone between days 2 and 20. Therefore, expression of *CO* from the CaMV 35S promoter is sufficient to overcome the delay in flowering caused by SDs, indicating that transcriptional regulation of *CO* is an important determinant of the photoperiodic control of flowering. In the experiments shown in Fig. 2, dexamethasone was

supplied to the plants repeatedly (see Methods); however, single applications to 8-day-old SD-grown plants had the same effect on flowering time as repeated applications. *CO* expression might therefore be required only transiently to trigger flowering, or after a single application the dexamethasone might persist in plant tissues maintaining *CO* expression.

In addition to accelerating flowering of *CO:GR* plants, dexamethasone treatments also affect leaf and shoot development. The most severe effects were caused by treating plants early in development. Activation of *CO:GR* with dexamethasone tended to reduce the number of cauline leaves and flowers produced on the primary shoot, and to reduce the size of rosette leaves and the length of the main stem (Figs 1 and 2). These effects are probably

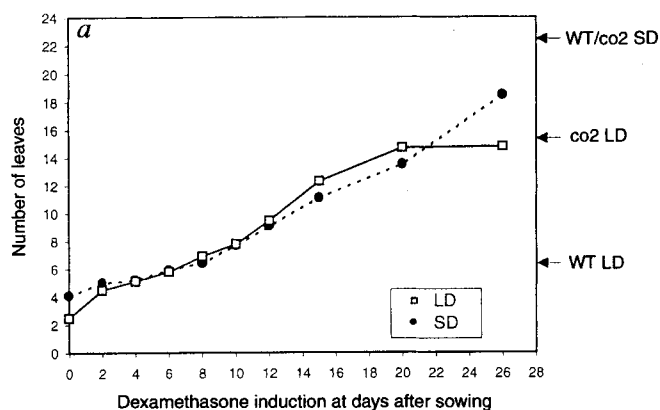
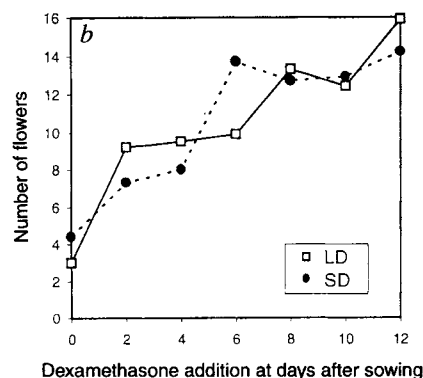


FIG. 2 Induction of flowering with dexamethasone at different times during development. a, The total number of leaves (rosette leaves plus cauline leaves on the primary stem) was counted when the plants were fully flowering. The numbers of leaves produced by control plants are indicated on the right. The developmental time of flowering of *Arabidopsis* is



accurately measured by determining the number of leaves formed before the development of flowers⁹. b, Number of flowers produced by plants treated with dexamethasone between d0 and d12. Wild-type plants form ~30 flowers under long days.

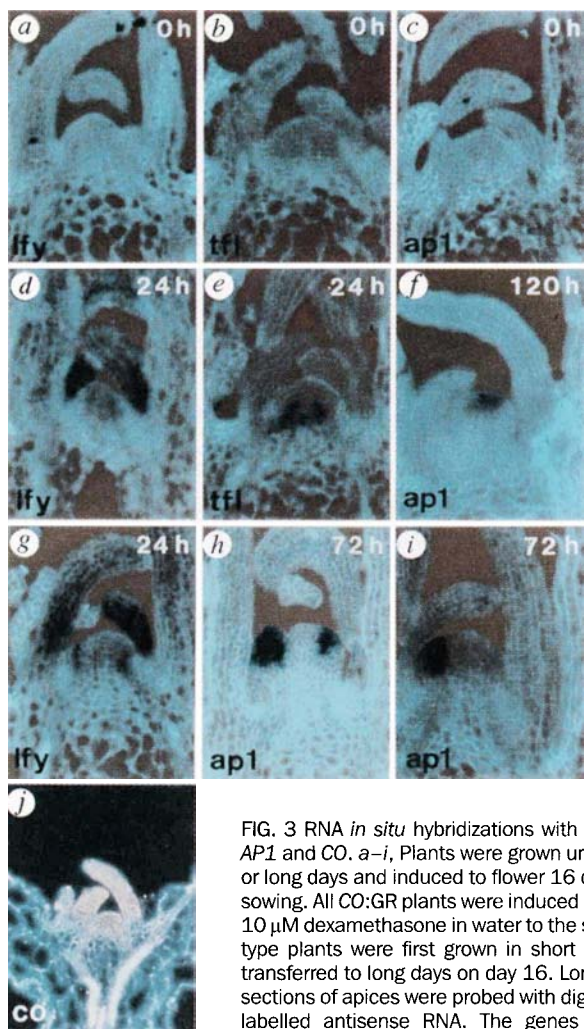


FIG. 3 RNA *in situ* hybridizations with *LFY*, *TFL*, *AP1* and *CO*. *a-i*, Plants were grown under short or long days and induced to flower 16 days after sowing. All *CO:GR* plants were induced by adding 10 μ M dexamethasone in water to the soil. Wild-type plants were first grown in short days and transferred to long days on day 16. Longitudinal sections of apices were probed with digoxigenin-labelled antisense RNA. The genes used as probes are indicated in each panel. The RNA signal is purple on a light-blue tissue background. Plant material was collected just before (0 h) and at different times (24, 72 and 120 h) after addition of dexamethasone (*d, e, f, h*) or movement from short days to long days (*g, i*). (*a-f*), *CO:GR* plants grown under short days. *h*, *CO:GR* plants grown under long days. *g, i*, Wild-type plants grown under short days and shifted to long days. *j*, An 8-day-old wild-type plant grown under long days. The *CO* gene was used as probe.

due to the high level of expression of *CO*, and are the converse of those caused by reducing *CO* function in *co* mutants^{9,10}. Wild-type plants grow indeterminately, continuing to form flowers on the main stem indefinitely. The *TERMINAL FLOWER* (*TFL*) gene prevents the conversion of the shoot meristem into a determinate floral primordium¹³, and represses the transcription of *LFY* and *AP1* within the shoot meristem¹⁵. However, plants in which *CO:GR* was activated by dexamethasone frequently terminated shoot development prematurely, with the formation of a carpeloid structure at the end of the primary shoot (Fig. 1m). *TFL* is expressed normally in induced *CO:GR* plants (see later), suggesting that expression of *CO* from the CaMV 35S promoter can overcome the effect of *TFL* and cause the ectopic activation of genes required for carpel development within the shoot meristem.

The floral meristem-identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) act early in floral development^{1,2} and are required for the proper development of floral organs, whereas the *TERMINAL FLOWER* (*TFL*) gene is expressed in the shoot meristem during flowering (D. Bradley, R. Carpenter and E. Coen, unpublished results). In LD-grown wild-type plants, *CO* is transcribed during vegetative development in the shoot meristem and in the first pair of vegetative leaves 8 d after sowing (Fig. 3j).

CO transcript is therefore present before *LFY* and *AP1* are expressed in floral primordia^{1,2}. In addition, *CO* transcript is more abundant in LD-grown than SD-grown seedlings¹⁰ and floral development is initiated rapidly after shifting plants from SDs to LDs¹⁴. The expression pattern of *CO* is therefore consistent with it acting in a pathway that promotes the expression of floral meristem-identity genes in response to LDs. However, in wild-type plants *CO* mRNA is extremely rare and therefore differences in the abundance of the transcript are difficult to quantify and to correlate with the expression of the floral meristem-identity genes. We therefore used dexamethasone treatments of *CO:GR* plants to test the effect of *CO* expression on *AP1* and *LFY* transcription and compared this with the effect of exposing wild-type plants to LDs. Sixteen-day-old *CO:GR* plants grown under SDs or LDs were treated with dexamethasone, and wild-type plants of the same age were shifted from SDs into LDs. Apices were harvested from the three populations just before the inductive treatment (time 0), and then at 24, 48, 72 and 120 h after treatment. As expected, transcripts of *AP1*, *LFY* and *TFL* were absent from time-zero control plants (Fig. 3a-c).

The transcripts of *LFY* and *TFL* were present in *CO:GR* plants 24 h after application of dexamethasone irrespective of whether the plants were grown in SDs or LDs (Fig. 3d, e). The patterns of transcription of these genes were similar to that in wild-type plants (Fig. 3; D. Bradley, R. Carpenter and E. Coen, unpublished results). Furthermore, in wild-type plants shifted from SDs to LDs, *LFY* transcript was detected 24 h after exposure to LDs. Thus in SD-grown plants, *LFY* transcription responds as rapidly to *CO* activity as to exposure of plants to LDs. The *AP1* transcript was present 72 h after treatment of LD-grown *CO:GR* plants (Fig. 3h), or 72 h after wild-type plants were shifted from SDs to LDs (Fig. 3i). In response to LDs, the *AP1* transcript therefore appears after the *LFY* transcript, which is in agreement with genetic data demonstrating that *AP1* acts after *LFY* in conferring floral meristem identity^{3,4}. However, in SD-grown *CO:GR* plants, there was a further delay in the appearance of the *AP1* transcript as it was not detected until 120 h after treatment with dexamethasone (Fig. 3f). Therefore, *AP1* was expressed more slowly in response to *CO* activation than in response to LDs.

These data indicate that *CO* acts within a genetic pathway that activates the transcription of *TFL* and *LFY* in response to LDs (Fig. 4). However, this pathway is not sufficient to activate *AP1* as rapidly as does exposing plants to LDs. We propose that another flowering-time pathway is activated by LDs and is required to

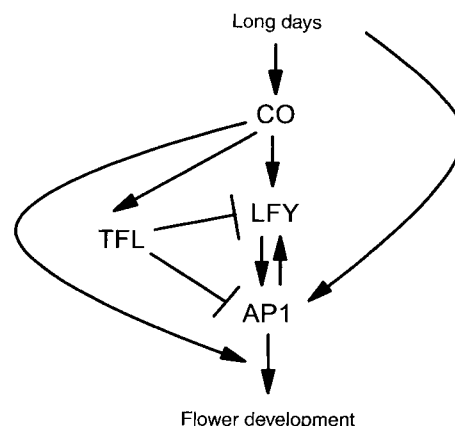


FIG. 4 A model for the role of *CO* in the regulation of floral meristem-identity gene expression. We propose that *CO* is activated in response to long photoperiods (days) that induce flowering, and that *CO* acts within a pathway that activates *LFY* and *TFL*. *CO* also regulates the competence of plants to respond to floral meristem-identity gene expression. *TFL* is a negative regulator of *LFY* and *AP1* transcription within the shoot meristem. Rapid activation of *AP1* transcription in response to long photoperiods requires another pathway in addition to that containing *CO*.

promote *AP1* transcription. Furthermore, *CO* is probably involved in a genetic pathway that acts after *LFY* and *AP1* (Fig. 4). Plants exposed to appropriate photoperiods only flower if the shoot meristem is competent to respond to the inductive signal formed in the leaves¹⁵; this observation was extended with the proposal that expression of *LFY* from the CaMV 35S promoter was not sufficient to promote flower development in very young SD-grown plants because of a delay in the competence of the meristem to respond to *LFY* (ref. 3). Dexamethasone-treated *CO:GR* plants form flowers very early under SDs (Figs 1, 2), indicating that *CO* not only promotes *LFY* expression, but might also alter the competence of the meristem to respond to *LFY*, either as a direct consequence of the presence of the *CO* protein or because *CO* alters the expression of other genes that enhance the response to *LFY*. □

Methods

Construction of the *CO:GR* fusion. The coding sequence of *CO* was amplified by use of the polymerase chain reaction from first-strand cDNA using the primers COGR3 (GGGGGATCCGCAATGAAGGAACAATCCCATATC) and COGR5 (GGGGTCTAGAGATGTTGAACAAGAGTAACG). This introduces an *Xba*I site at the 5' end and a *Bam*HI site at the 3' end of the *CO* open reading frame. This fragment was inserted into the vector pBI-ΔGR (ref. 11), creating a translational fusion of *CO* with the hormone-binding domain of the rat glucocorticoid receptor (amino acids 508–795). This construct was introduced into *Arabidopsis* plants carrying the *co-2* mutation by *Agrobacterium*-mediated root transformation. Transgenic lines were selected on medium containing kanamycin and made homozygous for the transgene.

Flowering-time tests. Seeds were soaked in water for 4 days at 4 °C before sowing on soil. Plants were grown in controlled environment rooms with 16 h light (long days) or 10 h light (short days), as described¹⁰. Dexamethasone was dissolved in ethanol, and 10 μM dilutions made in water were applied to the soil every second day. For each time point, 10 plants were analysed and average leaf

and flower numbers were calculated. The variation between plants of one time point was less than 10%.

In situ hybridizations. Methods for digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridization have been described¹⁶. *TFL* is a homologue of the *Antirrhinum majus* gene *Centroradialis*¹⁷; the *TFL* probe was made from plasmid pJAM2045, which contains an internal fragment of ~500 bp of *TFL* subcloned into pGEMT (provided by D. Bradley, R. Carpenter and E. Coen). The *LFY* probe was made using plasmid pDW122 (ref. 1). The *AP1* probe was made using pKY89 (ref. 4).

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Initiation of vertebrate left-right axis formation by maternal *Vg1*

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In the development of the three-dimensional vertebrate body plan, the left–right axis is linked to the dorsoventral and anteroposterior axes^{1,2}. In humans, altered left–right development results in severe cardiovascular and visceral abnormalities in individuals and in conjoined twins^{3,4}. Although zygotically transcribed genes that are asymmetrically expressed have been identified^{5–8}, the mechanism by which left–right asymmetries are established during embryogenesis is unknown⁹. Here we show that the *Xenopus* maternal gene *Vg1*, a member of the TGF-β family of cell-signalling molecules which are implicated in dorsoanterior development¹⁰, initiates left–right axis formation. Altered expression of *Vg1* on the right side of 16-cell embryos or disruption of endogenous *Vg1* signalling on the left side randomizes cardiac and visceral left–right orientation and alters expression of *Xnr-1*, a nodal-related molecular marker for left–right development⁶. Furthermore, the orientation of the left–right axis in conjoined twins is dependent upon which cell-signalling molecule initiated twin formation and on whether the secondary axis is on the left or right side of the primary embryonic axis, implicating a molecular pathway leading to the formation of conjoined twins.

The cortical rotation in the first cell cycle of *Xenopus* embryos establishes the embryonic body axes¹¹. Perturbation of this micro-

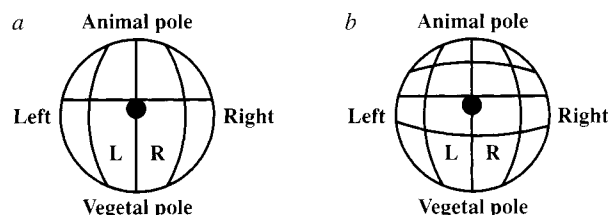


FIG. 1 Targeted ectopic gene expression in the left or right side of the Nieuwkoop centre. Dorsal view of a, 16-cell stage embryo, and b, 32-cell embryo, with the location indicated of the left (L) and right (R) vegetal cells that were injected with the RNAs listed in Table 1 and which contribute to the Nieuwkoop centre. Black dot represents Nile blue stain, through which point the cleavage plane passes that bisects left and right sides.

tubule-dependent rotation results in diminished dorsoanterior development¹¹ and randomization of left–right asymmetries¹. Before zygotic transcription begins, a group of cells in the vegetal pole of the embryo (Fig. 1; the ‘Nieuwkoop centre’) emit cell-signalling factors encoded by maternal genes that lead to mesoderm induction and dorsoanterior axis formation. Transplantation of dorsovegetal cells to the ventral side of an embryo¹¹ or injection of a certain messenger RNAs into ventral cells¹² results in the induction of a secondary embryonic axis and formation of conjoined twins. There are left–right asymmetries in the mesoderm-inducing capacity of the Nieuwkoop centre¹³, which might be explained by asymmetrical expression of the maternally encoded cell-signalling molecules that orchestrate axis formation. This early left–right asymmetry would then initiate subsequent asymmetries in zygotic gene expression, some of which have been characterized^{5–8}. To identify the molecular nature of the left–right asymmetry in the Nieuwkoop centre, purified mRNAs synthesized from complementary DNA clones of several genes implicated in dorsoanterior embryonic axis formation were microinjected

TABLE 1 BVg1 RNA, but not other dorsaling RNAs, randomizes left–right orientation

RNA injected	Amount injected	Left-injected % reversal (N)	Right-injected % reversal (N)	Ventral-injected % second axis (N)
BVg1	250–500 pg	10 (160)	52 (137)	36 (36)
BVg1-2cx	400 pg	3 (35)	3 (38)	ND
Vg1	1–4 ng	6 (84)	3 (86)	2 (65)
Activin	0.5–6 pg	4 (78)	3 (73)	22 (226)
Noggin	100–400 pg	3 (117)	0 (122)	64 (66)
Xwnt 8	10–50 pg	0 (72)	0 (76)	5 (37)
Xwnt 8	200 pg	9 (34)	8 (25)	95 (70)
Xwnt 11	400–1,600 pg	1 (99)	1 (113)	7 (42)
Siamois	100–500 pg	5 (160)	5 (156)	54 (39)
Water	8 nl	0 (18)	0 (22)	0 (18)
LacZ	500 pg	0 (39)	0 (38)	0 (17)
Uninjected	NA	1 (495)	1 (495)	0 (495)

Embryos were injected into indicated dorsovegetal cell at the 16-cell stage (Fig. 1) and scored for the rate of cardiac left–right reversal, or into ventrovegetal cell and scored for secondary axis formation. *N*, number scored; ND, not determined; NA, not applicable.

into either left or right vegetal cells (Fig. 1). If there is a left–right asymmetry in the expression of one of these genes, ectopic expression of the gene in the contralateral side, or inhibition of the signalling pathway on the same side, should override the endogenous asymmetry and result in altered left–right development.

Endogenous maternal Vg1 RNA is localized to the vegetal pole of developing oocytes and so is present in the Nieuwkoop centre¹⁴. However, injection into ventral cells does not induce secondary axes^{10,15} and injection of Vg1 RNA into either dorsovegetal cell had no effect on left–right development (Table 1). The inability of Vg1 RNA to induce a secondary axis may be due to a tight regulatory step in Vg1 protein processing in which the precursor form of Vg1 is cleaved into the mature Vg1 protein^{10,15,16}. BVg1 RNA encodes a protein in which the pro-region and the processing site of a bone morphogen protein (BMP-2) is fused to the mature peptide region of Vg1, ensuring that mature Vg1 protein is processed for cell signalling¹⁰. Injection of BVg1 RNA into the right dorsovegetal cell (that is, into the right-hand side of the Nieuwkoop centre; Fig. 1) resulted in randomization of cardiac and visceral left–right asymmetry (Fig. 2b and Table 1). Like other treatments later in embryogenesis that perturb left–right development¹⁷, the left–right orientations of the heart and the gut segregate independently of each other when underlying left–right axis information has been altered. Injection of BVg1 RNA into the left dorsovegetal cell did not significantly alter left–right development (Fig. 2a and Table 1). Injection of other RNAs

that encode factors implicated in dorsoanterior development had no effect on left–right development, although these RNAs were biologically active and capable of forming secondary axes at the expected rates¹² when injected into ventral cells (Table 1). Similarly, BVg1-2cx, a mutant in which elimination of two cysteines in

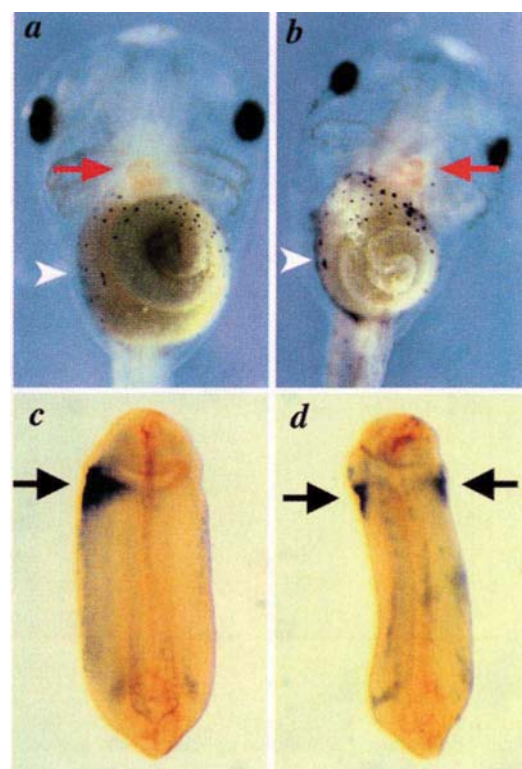
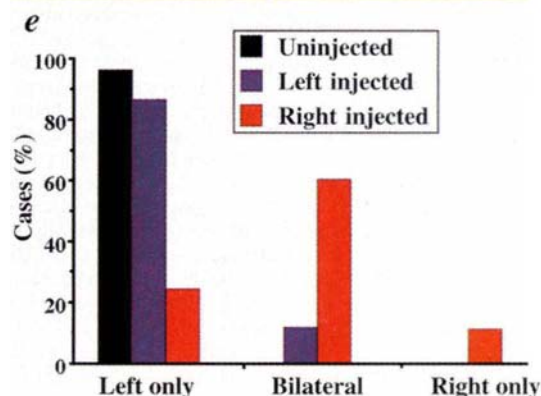


FIG. 2 Ectopic expression of BVg1 RNA in the right dorsovegetal cell randomizes the orientation of left–right asymmetries and induces ectopic expression of Xnr-1 in right lateral plate mesoderm. *a*, Embryo injected at 16-cell stage in the left dorsovegetal cell with BVg1 RNA had normal left–right orientation of the heart (red arrow) and viscera (white arrowhead). *b*, Embryo injected in the right dorsovegetal cell with BVg1 RNA had heterotaxia, reversed cardiac left–right orientation and normal visceral orientation. Embryos with reversed cardiac left–right orientation displayed reversed visceral orientation in 55% of the cases (*N* = 20). *c*, Embryo (at stage 24) injected with BVg1 in the left dorsovegetal cell displays normal Xnr-1 expression in left lateral plate mesoderm⁸ (black arrow). *d*, Embryo (stage 26) injected with BVg1 in the right cell shows bilateral Xnr-1 expression: normal expression in the left lateral plate⁸ and a mirror image of this expression in the right lateral plate. Xnr-1 expression becomes more restricted in older embryos⁸. *e*, Graph of the percentage of cases displaying the indicated Xnr-1 expression patterns in uninjected (black, *N* = 27), left-injected (blue, *N* = 59) and right-injected (red, *N* = 53) embryos. 'Left only' refers to embryos in which Xnr-1 is expressed in the left but not right lateral plate mesoderm; 'bilateral' refers to embryos in which Xnr-1 is expressed in both the left and right lateral plate mesoderm; 'right only' refers to embryos in which Xnr-1 is expressed only in the right, not left, lateral plate mesoderm.



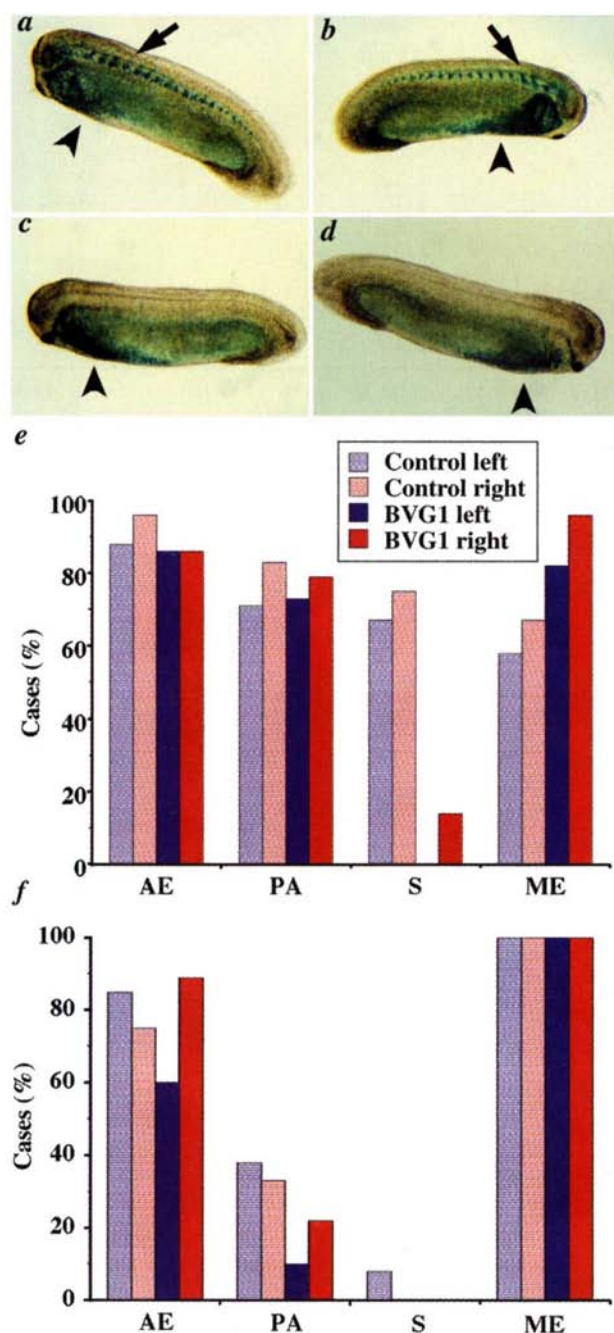


FIG. 3 Alteration in fate maps is not correlated with altered left-right development. Lateral views of tailbud stage (stage 26–28) embryos injected into the left (a, c) or right (b, d) dorsovegetal cell at the 16-cell stage with nuc- β -galactosidase-encoding RNA alone (a, b) or mixed with BVG1 RNA (c, d). Somite (arrows) and anterior endoderm (arrowheads) stainings are indicated. e, Injections at the 16-cell stage. The percentage of embryos staining for β -galactosidase in the anterior endoderm (AE), pharyngeal arches (PA), somites (S) and middle endoderm (ME), shown for β -galactosidase RNA alone in the left (blue horizontal bars, $N = 24$) or right (red horizontal bars, $N = 24$) cell or β -galactosidase RNA mixed with BVG1 RNA in the left (solid blue, $N = 22$) or right (solid red, $N = 28$) cell. In only 9% of cases, lineage label injected on one side exclusively labelled anterior endoderm on the contralateral side, regardless of whether injections were on the left or right, indicating that the accuracy of predicting the midline and targeting cell injections was greater than 90%. f, Injections at 32-cell stage, shown for β -galactosidase RNA alone in the left (blue horizontal bars, $N = 13$) or right (red horizontal bars, $N = 12$) cell, or mixed with BVG1 RNA in the left (solid blue, $N = 10$) or right (solid red, $N = 9$) cell.

the mature Vg1 protein sequence prevents protein-precursor processing¹⁰, had no effect on left-right development (Table 1). Thus, alteration of left-right axis formation is specific to BVG1 injection into the right dorsovegetal cell, suggesting that left-right axis formation is initiated by the regulated protein processing of maternal Vg1. The production of mature Vg1 by BVG1 RNA injection can override the endogenous constraints of Vg1 protein processing to alter left-right development, whereas exogenously introduced Vg1 and BVG1-2cx cannot be processed¹⁰ and so did not alter left-right development.

To test whether inhibition of endogenous Vg1 signalling alters left-right development, RNA encoding a dominant-negative truncated activin receptor (tAR), which blocks Vg1 signalling in mesoderm induction assays¹⁸, was injected into 16-cell embryos (Fig. 1). Embryos injected in either the left side or right side had normal dorsoanterior development (data not shown). However, left-side-injected embryos had cardiac reversal rates (51%, $n = 41$, at 2 ng) that were significantly higher ($P < 0.01$) than those of right-side-injected embryos (17%, $n = 29$). Although this dominant-negative receptor also interferes with receptor-mediated pathways of the other ligands^{18,19}, these results concur with the conclusion that endogenous Vg1 signalling on the left side is necessary to initiate left-right axis formation.

Xnr-1, a *Xenopus* nodal-related gene and another member of the TGF- β family, is normally expressed in the left lateral plate mesoderm⁸, which places it in juxtaposition to the cardiac mesoderm and visceral primordia that will form left-right asymmetric organs. Embryos injected with BVG1 RNA in the left side of the Nieuwkoop centre had normal cardiac left-right orientation and normal asymmetric (left-sided) Xnr-1 expression patterns (Fig. 2c). Embryos injected with BVG1 in the right side of the Nieuwkoop centre displayed bilaterally symmetric Xnr-1 expression (Fig. 2d), and injection of tAR in the left side altered Xnr-1 expression (data not shown), suggesting that Vg1 induces Xnr-1 expression in the right lateral plate mesoderm. Thus, altered expression patterns of Xnr-1 (Fig. 2e) are correlated with altered cardiac and visceral left-right orientation in *Xenopus*, as they are for nodal-related genes in chicken⁵ and mouse^{6,7} embryos. These results indicate that the nodal-related, zygotically expressed gene *Xnr-1* is downstream of maternal Vg1 and upstream of the specification of left-right orientation. A prediction now being tested is that both ectopic expression of Vg1 and Xnr-1 on the right side and greatly diminished activity of both pathways on the left side are required for 100% reversal rates.

Possible mechanisms by which BVG1 injection into the right dorsovegetal cell might alter left-right development that have been ruled out include perturbations of dorsoanterior development¹, diminished notochord development^{1,2}, and alterations in cell-fate maps. BVG1-injected embryos displayed normal dorsoanterior development (Figs 2 and 3) and normal notochord development (see Supplementary Information). Dorsovegetal cells in 16-cell embryos (Fig. 1a) injected with BVG1 decreased their contribution to somites and increased their contribution to endoderm (Fig. 3e). This was not unexpected, because animal cap ectoderm expresses endoderm makers when injected with BVG1

TABLE 2 Left-right axis in conjoined twins

RNA injected	2° axis on left		2° axis on right	
	N	% Reversal	N	% Reversal
Activin	30	13	28	54
Noggin	13	62	27	0
BVG1	26	15	14	14

Left-right orientation in twins is dependent both on the cell-signalling molecule used to generate twins and on the position of the secondary axis. Position of secondary axis and cardiac left-right orientation were scored. *N*, number of conjoined-twin embryos examined.

(ref. 20). However, injection of BVg1 on the left and right sides produced statistically identical alterations in the fate maps, indicating that cell-fate changes are not correlated with the randomization of left-right asymmetries. Furthermore, BVg1 RNA injections into dorsovegetal cells of a 32-cell stage embryo (Fig. 1b) did not alter the endoderm-specific fate of either injected cell (Fig. 3f). Cardiac reversal rates differed between right injections (26% reversal, $N = 35$) and left injections (0% reversal, $N = 38$); the lower reversal rate in embryos injected at the 32-cell stage compared to those injected at the 16-cell stage (Table 1) was probably because only half as many progeny were expressing BVg1, or BVg1 expression was delayed by one cell cycle. These results indicate that changes in fates of the injected cells did not cause randomization of left-right asymmetries or the induction of bilaterally symmetric expression of Xnr-1.

Disruption of normal organ laterality within an individual embryo is analogous to cases of heterotaxia in humans⁴, which occurs in monozygotic twins and in conjoined twins³. Some of the *Xenopus* conjoined twins induced by injections of RNAs into ventral cells (Table 1) were analysed for left-right axis formation (Table 2). As complete dorsoanterior axis formation¹ and normal notochord development^{1,2} are required for normal left-right development, and most induced secondary axes do not form complete dorsoanterior axes, only the primary embryonic axis in *Xenopus* twins was analysed for left-right development. Surprisingly, left-right orientation with respect to the primary embryonic axis was dependent on which cell-signalling molecule was used to generate a secondary axis and on whether the secondary axis was to the left or right side of the primary embryonic axis (Table 2). In conjoined twins generated by activin RNA injections, embryos with a secondary axis to the right of the primary axis had a significantly higher rate of left-right reversal than the rate of left-right reversal in embryos with a secondary axis on the left. In contrast, in conjoined twins generated by injection of *noggin* RNA, embryos with a secondary axis on the left of the primary axis had a significantly higher rate of left-right reversal than the rate of left-right reversal in embryos with a secondary axis on the right. Secondary axes induced by BVg1 injections had low effects on left-right orientation within the primary axis, confirming that the effects on left-right development of BVg1 ectopic expression are confined to tightly localized expression in the right dorsal quadrant of the Nieuwkoop centre (Table 1). Thus, the orientation of the left-right axis in conjoined twins was dependent both on the signalling molecule used to instigate twinning and on the spatial relationship of the twins. In human conjoined twins, the twin arising from secondary axis formation cannot be ascertained, although twins on the right side display randomization of left-right orientation³. These observations are consistent with the postulate that the left twin in conjoined human twins is formed

by a secondary axis driven by ectopic activation of a *noggin*-related pathway. In amphibian conjoined twins caused by medial constriction of embryos, left-right orientation in the right twin is randomized²¹. This is consistent with the prediction that the embryo formed from the left side contains adequate Vg1 signalling for left-right development, and that the embryo formed from the right side, separated from Vg1 signalling, becomes randomized.

These results suggest that the molecular pathway for vertebrate left-right development is initiated by differential protein processing of maternal Vg1. Although it is not known when Vg1 processing occurs, maternal Vg1 RNA and precursor protein are present in *Xenopus* unfertilized eggs and blastula before zygotic transcription begins and Vg1 precursor protein is synthesized through late gastrula and is present through neurula stages^{10,14,15,22,23}, coincident with the developmental specification of Xnr-1 (J.L.L., M. C. Danos, H.J.Y., manuscript submitted) and cardiac² left-right orientation. All other known genes in the pathway in chick, mouse, frog and zebrafish are zygotically transcribed genes^{2,5-8,24}. Overexpression of mature Vg1 protein on the right side of blastula-stage embryos induces ectopic Xnr-1 expression on the right side, leading to embryos that have bilaterally symmetric Xnr-1 expression patterns, and randomizes both cardiac and visceral left-right orientation. These results imply that endogenous mature Vg1 is more strongly expressed on the left side of blastula, probably as a result of differential regulation of Vg1 protein processing. Inhibition of the endogenous Vg1 signalling pathway by expressing a dominant-negative mutant receptor on the left side also results alters left-right development. This places endogenous Vg1 signalling upstream in the molecular pathway that leads to induction of Xnr-1 asymmetric expression and to the left-right asymmetric development of the heart and gut. □

Methods

Fertilized *Xenopus* embryos were tilted in 4% Ficoll in Sorenson's solution and stained with a Nile blue dot to identify the future dorsal side¹¹. Embryos were selected for normal cleavage patterns and cleavage through the Nile blue dot at the dorsal midline at the 16-cell stage. Embryos were placed in 4% Ficoll and injected with RNAs that had been synthesized *in vitro*²⁵ (Stratagene) and resuspended in water. The sources of cDNA clones are given in ref. 12. The activity of each RNA was titrated. The concentrations listed in Table 1 were used for analysis of left-right axis formation by injection into dorsovegetal cells in 16-cell and 32-cell injections and for induction of conjoined twins by injection into a ventral vegetal cell in 8-cell embryos. Left-right asymmetries of the heart and viscera were scored in anaesthetized embryos at stage 42–43 as described¹⁷. For Xnr-1 pattern analysis²⁶, embryos were fixed at stage 24–26 and processed for whole-mount *in situ* hybridization²⁷ with digoxigenin-labelled antisense RNA prepared from Xnr-1 cDNA²⁶. For fate mapping, embryos were injected at the 16-cell or 32-cell stage, fixed at tailbud stages (stage 26–28) and assayed by whole-mount staining for nuclear-localized β -galactosidase activity²⁸.

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CORRESPONDENCE and requests for materials to H.J.Y. (e-mail: yost001@maroon.tc.umn.edu).

Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V

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COAGULATION factor V is a critical cofactor for the activation of prothrombin to thrombin, the penultimate step in the generation of a fibrin blood clot^{1,2}. Genetic deficiency of factor V results in a congenital bleeding disorder (parahaemophilia)³, whereas inheritance of a mutation rendering factor V resistant to inactivation is an important risk factor for thrombosis^{4,5}. We report here that approximately half of homozygous embryos deficient in factor V ($Fv^{-/-}$), which have been generated by gene targeting, die at embryonic day (E) 9–10, possibly as a result of an abnormality in the yolk-sac vasculature. The remaining $Fv^{-/-}$ mice progress normally to term, but die from massive haemorrhage within 2 hours of birth. Considered together with the milder phenotypes generally associated with deficiencies of other clotting factors^{6,7}, our findings demonstrate the primary role of the common coagulation pathway and the absolute requirement for functional factor V for prothrombinase activity. They also provide direct evidence for the existence of other critical haemostatic functions for thrombin in addition to fibrin clot formation, and identify a previously unrecognized role for the coagulation system in early mammalian development.

The gene-targeting strategy used to generate factor-V-deficient mice is shown in Fig. 1a. Southern blot screening of neomycin (*neo*)-resistant embryonic stem (ES) colonies indicated targeting efficiency of ~10%. Successful targeting results in replacement of factor V exons 8–11 by a *neo* cassette, introducing a frameshift mutation near the amino terminus of factor V. F_1 mice heterozygous for the targeted allele ($Fv^{+/-}$) were intercrossed to generate homozygous $Fv^{-/-}$ progeny. Southern blot analysis confirmed the expected structure of the targeted *Fv* locus (Fig. 1b, c).

Factor-V-deficient animals were immediately evident at birth and generally died within 2 hours of massive intra-abdominal haemorrhage (Fig. 2). Cutaneous bleeding, particularly over the head, was occasionally evident, and scattered microscopic haemorrhages were detected in a variety of tissues. No other gross or microscopic abnormalities were identified. One of 60 homozygous-null pups survived until day 10 and a second pup died immediately following a tail biopsy at day 14. Blood present intra-abdominally was uniformly unclotted and was completely deficient in factor V activity (Fig. 1d). $Fv^{+/-}$ mice appeared to be entirely normal and haemostasis was normal after tail biopsy. Factor V activity in the plasma of $Fv^{+/-}$ mice was about half that in wild-type homozygotes (Fig. 1d).

The fatal neonatal haemorrhage in $Fv^{-/-}$ mice was unexpected, given the much milder phenotype associated with human factor V deficiency³. The severity of the defect in these animals also contrasts with the phenotype of afibrinogenemic humans⁸ and fibrinogen-knockout mice⁹; both generally survive into adulthood. These results support the idea of further haemostatic functions for thrombin activation in addition to formation of the fibrin blood clot, probably related to the presumed role of thrombin in platelet activation^{9,10}. They show that factor V is an essential component of the prothrombinase complex and that factor Xa has little or no prothrombinase activity *in vivo* in the absence of factor V, con-

sistent with previous *in vitro* kinetic studies¹¹. They also indicate that there is probably no significant alternative pathway for the generation of thrombin.

Although thrombin activity may be essential for platelet function in haemostasis, mice that completely lack platelets, as a result of targeted disruption of the haematopoietic specific transcription

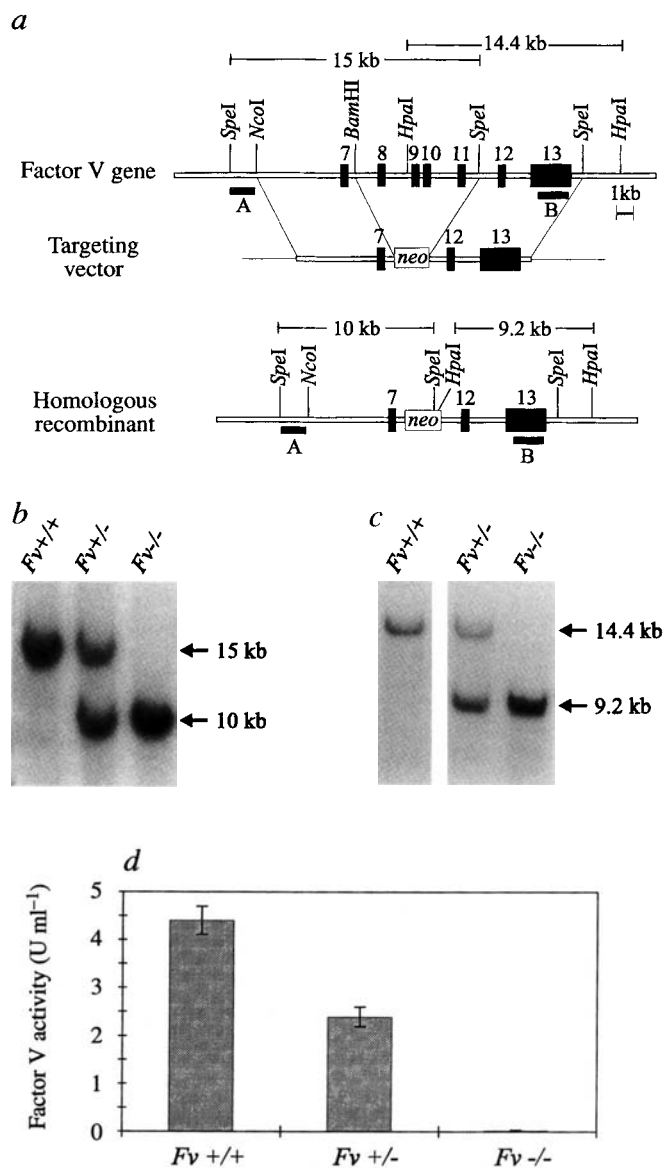


FIG. 1 Targeting of the *Fv* gene by homologous recombination. a, Structure of exons 7–13 of the murine *Fv* gene and targeting vector. The targeting vector contains a *neo* cassette, which replaces murine exons 8–11 flanked by ~6.6 kb homologous 5' and 3' arms. The *neo* cassette is driven by the *pgk* promoter²⁴. The predicted product of successful homologous recombination is shown at the bottom. The location of hybridization probes A and B, used to detect successful targeting, are indicated. The mutant allele carries additional *SpeI* and *HpaI* sites within the *neo* cassette. Southern blot analysis demonstrating the expected genomic structure at the 5' end of the locus is shown in b and the 3' end in c. Genomic DNA prepared from tail biopsies of $Fv^{+/+}$, $Fv^{+/-}$ and $Fv^{-/-}$ mice was analysed by restriction digestion with *SpeI* and hybridization with probe A (b), or by restriction digestion with *HpaI* and hybridization with probe B (c). New genomic fragments of the expected size (10 and 9.2 kb) are seen in $Fv^{+/-}$ and $Fv^{-/-}$ mice and the normal allele (15 and 14.4 kb) is absent from $Fv^{-/-}$ mice. d, Plasma factor V functional activity in factor-V-deficient mice. Values are the mean $\pm$ s.d. of determinations in 5 ($Fv^{+/+}$ and $+/-$) or 3 ($Fv^{-/-}$) animals. No factor V activity was detectable in the $Fv^{-/-}$ plasma, although $Fv^{+/-}$ plasma contained about half the activity of $Fv^{+/+}$ plasma.

factor NFE-2 (ref. 12), also bleed less severely than observed here. The profound haemorrhage exhibited in $F_v^{-/-}$ mice probably results from the simultaneous interruption of both platelet activation and fibrin formation.

DNA analysis of ~300 progeny mice at term, derived from an intercross of $F_v^{+/-}$ mice, identified a highly significant decrease in the number of $F_v^{-/-}$ progeny, compared to the expected 25% frequency. Although the expected number of $F_v^{-/-}$ embryos were present at E10.5 and E9.5, a decrease was evident by E11.5, with highly significant differences at E15.5 and E18.5 (Table 1).

By E9.5, $F_v^{+/+}$ and $F_v^{+/-}$ embryos (and ~60% of $F_v^{-/-}$ embryos) had 20–25 somites, with a well developed heartbeat and yolk-sac circulation. However, 17/43 $F_v^{-/-}$ E9.5 embryos showed some degree of developmental delay, many having only 12–16 somites (Fig. 3). Anomalies of yolk-sac organization were also present in a number of null embryos at E9.5. In many null embryos, the yolk-sac circulation was sluggish and the yolk sac had a granular appearance, rather than the smooth surface typical of control embryos. On histological analysis, visceral yolk sacs from these embryos were strikingly abnormal (Fig. 3). By E9.5, the visceral yolk sac consists of an outer endodermal layer and inner mesodermal layer, with large vascular plexes containing haematopoietic stem cells, the blood islands. In 5/11 yolk sacs from null embryos examined histologically, the visceral endoderm appeared flattened, with few blood islands. The additional six null yolk sacs appeared to have slightly fewer haematopoietic precursors, but vascular plexes were present in the mesoderm.

Thus, complete deficiency of factor V results in an incomplete block to murine embryonic development, leading to loss of ~1/2 of $F_v^{-/-}$ embryos at E9.5–10.5, with the remaining animals continuing to develop to term. It is unclear why there is this dichotomy among animals with identical genotypes. The incomplete embryonic-lethal and perinatal haemorrhagic phenotypes were confirmed in animals derived from three independent ES clones (from two different established ES cell lines), excluding an unrelated second mutation or clone-specific effect. The pattern of lethality is not consistent with a genomic imprinting mechanism, and a potential sex-limited modification of the embryonic-lethal phenotype was also excluded by genotyping for the Y-chromosome-specific sequence *SRY*¹³. A contribution from genetic background differences among mouse strains, as reported for targeted deletion of the EGF receptor¹⁴, was excluded by analysis of $F_v^{+/-}$ mice derived from four successive backcrosses to C57BL/6J or backcrosses onto a pure 129Sv background (Table 1).

Two groups have described the targeted deletion of the tissue-factor gene (*TF*) in mice: although both report an embryonic-lethal phenotype between E8.5 and 10.5, Bugge *et al.*¹⁵ conclude that death of $TF^{-/-}$ embryos is secondary to early haemorrhage, whereas Carmeliet *et al.*¹⁶ propose that there is a defect in early blood vessel development. Our results are more consistent with the latter model. Specifically, detailed histological analysis of over

TABLE 1 Genotypes of progeny from $F_v^{+/-}$ intercrosses at varying stages

	Total	+/+	+/-	-/-	
Term pups	297	89 (30.0%)	176 (59.2%)	32 (10.8%)	$P < 1 \times 10^{-6}$
E18.5	109	37 (34%)	60 (55.0%)	12 (11.0%)	$P < 0.002$
E15.5	110	32 (29.1%)	65 (59.1%)	13 (11.8%)	$P < 0.01$
E11.5	72	20 (27.8%)	41 (56.9%)	11 (15.3%)	$P \sim 0.16$
E10.5	141	31 (22.0%)	76 (54.0%)	34 (24.0%)	$P > 0.5$
E9.5	137	36 (26.2%)	62 (45.3%)	39 (28.5%)	$P > 0.5$
C57BL6/J background					
Term pups	117	36 (30.8%)	69 (59.0%)	12 (10.3%)	$P < 0.002$
E18.5	50	20 (40.0%)	23 (46.0%)	7 (14.0%)	$P < 0.03$

About 1/2 of the $F_v^{-/-}$ embryos are lost after E10.5, with the remaining half surviving to term. 'C57BL6/J background' indicates the results of an intercross between $F_v^{+/-}$ mice derived from four successive backcrosses to C57BL6/J. In addition, the original chimaeric founders were backcrossed to 129Sv to produce $F_v^{+/-}$ mice on a pure 129Sv background. Analysis of an intercross between these 129Sv $F_v^{+/-}$ mice identified only 3/26 $F_v^{-/-}$ progeny at E18.5, consistent with the patterns observed on the C57BL6/J background, as well as the original intercross. To detect a potential sex-limited modification of the embryonic-lethal phenotype, 15 $F_v^{-/-}$ term pups were typed by PCR for the Y-chromosome-specific sequence *Sry*¹³. Eight pups were identified as male and seven as female, excluding a modifying effect of the sex chromosomes as the explanation for these unusual survival data. Statistical significance was calculated using the χ^2 test to compare the observed and expected frequencies.

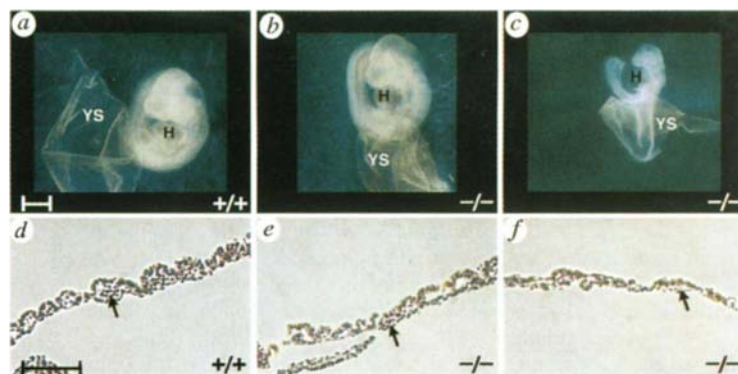
40 $F_v^{-/-}$ E9.5 embryos identified only occasional microscopic haemorrhages, most commonly in the mesenchyme of the face, and were strikingly different from the massive haemorrhage described in $TF^{-/-}$ embryos by Bugge *et al.*¹⁵.

A 50% loss at E9–10 occurs in mice homozygous for disruption of the thrombin receptor (*TR*) gene¹⁷, remarkably similar to our observations of $F_v^{-/-}$ mice, despite the lack of any haemostatic defect in fetal or adult $TR^{-/-}$ mice. Taken together, these results suggest that factor-V-dependent generation of thrombin and a subsequent signal through the thrombin receptor are both required at a critical step in early development, potentially related to yolk-sac vasculogenesis. Apparently, stochastic compensatory mechanisms permit this block to be overcome in approximately 1/2 of animals, with normal development proceeding past this

FIG. 2 Newborn progeny of $F_v^{+/-}$ intercross. The litter depicted here contained a single homozygote $F_v^{-/-}$ (middle animal in the lower row). Genotypes of the remaining six mice were two $F_v^{+/+}$ and four $F_v^{+/-}$. The single homozygote-null animal has suffered massive intra-abdominal haemorrhage and is a pale colour over the upper body because of blood loss.



FIG. 3 Embryos isolated at E9.5. The wild-type (+/+) embryo shown in *a* and the null (-/-) embryo in *b* have developed to the 20–24-somite stage, whereas the null embryo in *c* is developmentally delayed. The most common anomalies in these embryos were focal haemorrhages (14/43 null embryos), anomalous positioning of the first branchial arch, as well as cardiac and pericardial anomalies. Both developmentally delayed and 20–25-somite-stage null embryos were characterized by anomalies of the 'turning' process, in which the early murine embryo reverses its inverted U shape to adopt the dorsal curvature (C shape) typical of the day-10 embryo. Defects of axial rotations were observed in 23/43 null embryos and positional defects were also seen in 12/108 embryos identified as heterozygous (+/-) or wild-type (+/+). When examined histologically, *Fv*^{-/-} embryos had defective development of cardiac muscle: specifically trabeculation of the myocardium was delayed or absent. Small haemorrhages had occurred in the mesenchyme, particularly in the cephalic region. In these embryos, the mesenchyme was often abnormally condensed, and it was common to observe a lack of development of the posterior region. Histological section of a wild-type visceral yolk sac (*d*) illustrates the normal appearance of outer endodermal layer and blood islands containing haematopoietic stem cells (arrow) within the extraembryonic mesoderm. A similar pattern is seen in



yolk sacs isolated from the subset of null embryos that had developed to the 20–24-somite stage (*e*). In contrast, vascular channels (arrow) in yolk sacs from a population (5/11) of null embryos appeared to have collapsed (*f*). Scale bars: 500 μ m in *a*–*c*, and 100 μ m in *d*–*f*. H, heart; YS, yolk sac.

bottleneck. The previous demonstration of widespread thrombin receptor messenger RNA expression in mesenchymal cell populations at E9.5 (ref. 18) is consistent with this model. The well known teratogenic effect of warfarin, an inhibitor of several coagulation factors, including factor Xa and thrombin¹⁹, may perhaps occur through a related mechanism^{20,21}. Although the thrombin receptor is abundantly expressed in the early embryo, prothrombin gene expression has not been detected in the mouse embryo before E12.5 (ref. 18), suggesting that the target for factor V in early development may be maternally derived prothrombin, very low levels of fetal prothrombin, or another as-yet unknown protease.

Other coagulation-factor deficiencies in mouse and humans, including those of factors VIII and IX (found in haemophilias A and B), and afibrinogenemia, are not associated with death of the embryo^{6–8,22}. In addition, the moderately severe bleeding phenotype of human factor V deficiency (parahaemophilia)³ sharply contrasts with the dramatic early-lethal phenotype we find in mice lacking factor V. Based on these observations, we suggest that complete factor V deficiency in humans may also be an early-embryonic-lethal condition. Consistent with this, nearly all reported cases of human factor V deficiency are associated with some degree of residual factor V activity and no *FV* gene deletions or null mutations have been identified in patients. These observations also suggest that complete deficiency of other components of the common coagulation pathway, such as factor X or thrombin, may likewise lead to lethal neonatal phenotypes. □

Methods

Factor V gene targeting. λ phage clones spanning ~30 kb of mouse *Fv* genomic sequences were previously isolated from a 129Sv genomic library

(Stratagene) and the structure of exons 7–12 characterized (J.C. *et al.*, manuscript in preparation). The targeting vector was constructed by assembling a 6.7-kb *NcoI*–*Bam*HI 5' *Fv* genomic fragment, a 1.4-kb *Bam*HI–*Xba*I fragment containing a neomycin (*neo*) expression cassette driven by the *pgk* promoter, and a 6.6-kb *SpeI*–*SpeI* 3' fragment of the *Fv* gene, all cloned into the plasmid vector pSL301 (Invitrogen). The targeting vector was linearized with *Sfi*I, introduced into the 129Sv-derived D3 (from T. Doetschman) and CJ7 (ref. 23) ES cell lines by electroporation, and stable transfectants selected as described²⁴. Individual ES clones were screened for homologous targeting by Southern blot analysis using probes A and B (Fig. 1). Ten of 100 *neo*-resistant ES clones carried the expected allele, for a targeting efficiency of 10%. By Southern blotting, each targeted clone contained only a single *neo* gene insertion at the expected site (data not shown). Eight independent ES clones (7 from D3 and 1 from CJ7) were injected into C57BL/6J blastocysts as described²⁴ and the resulting chimaeric males were bred to C57BL/6J females to generate *Fv*^{+/-} offspring.

Determination of factor V clotting activity. Whole blood from anaesthetized *Fv*^{+/-} and *Fv*^{-/-} mice was collected into sodium citrate (0.0129 M final) by cardiac puncture at 6 weeks of age. *Fv*^{-/-} blood was collected directly from the abdominal cavity of newborn *Fv*^{-/-} mice. Clotting was assayed as described (J. Cui *et al.*, manuscript in preparation) using factor-V-deficient human plasma. Units of factor V activity are defined relative to human factor V.

Genotyping by PCR. Genotypes of mice were established using DNA prepared from tail biopsies analysed by either Southern blotting as already described or by polymerase chain reaction (PCR). In the PCR assay, the wild-type and targeted *Fv* alleles were distinguished using primers specific for exon 10 of the murine *Fv* gene (5'ACGATCAGACCACTCAACCC' and 5'CCTGTAAACGTCCACATCAC3') and the *neo* gene (5'GGACTGGCTGCTATTGGGCGAAGTG3' and 5'GAAGAACTCGTCAA GAAGCGCATAGAGG3').

Histological analysis. Embryos were photographed at autopsy, fixed in 1% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature, embedded in paraffin and sectioned at 6 μ m (ref. 25). Sections were stained with haematoxylin and eosin and examined and photographed using a Leitz Orthoplan photomicroscope. Portions of yolk sacs or of embryos were removed for PCR genotyping.

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Synpolydactyly in mice with a targeted deficiency in the *HoxD* complex

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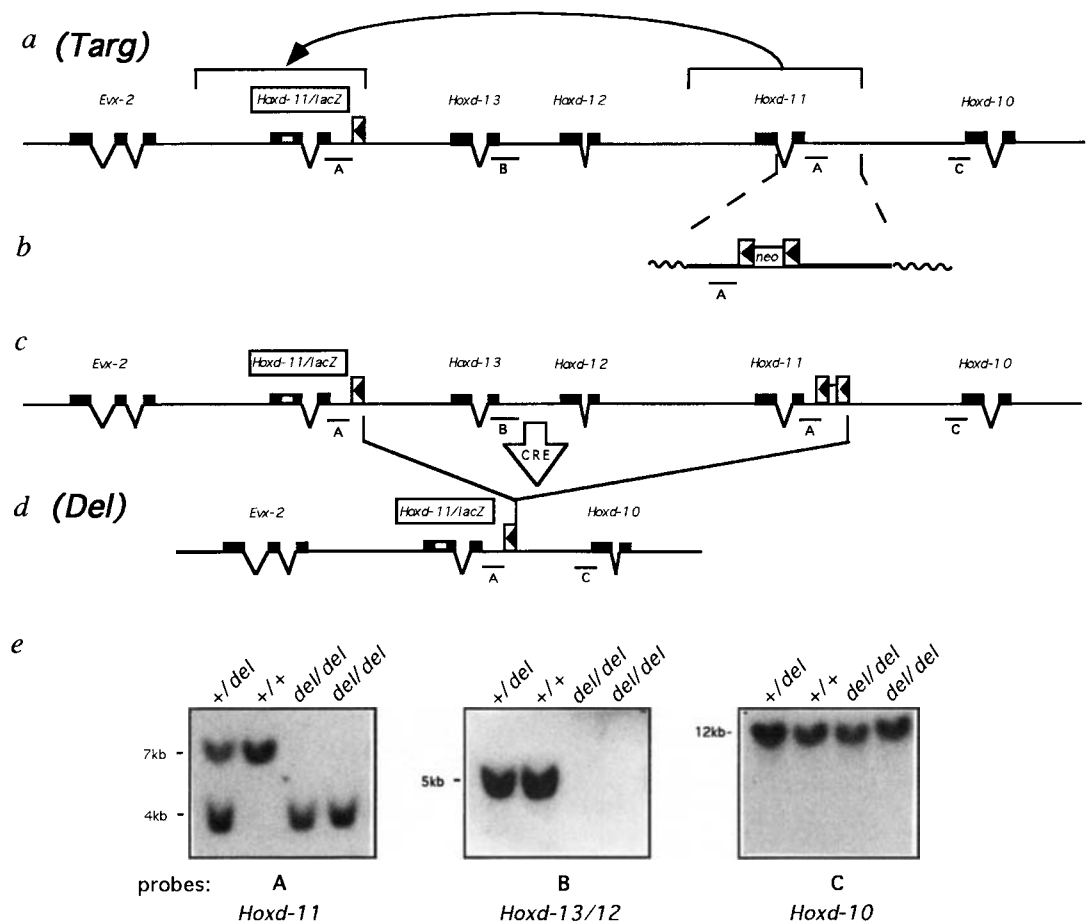
THE morphogenesis of mammalian digits requires the function of several genes of the *HoxD* complex during development of limb buds¹⁻⁴. Using embryonic stem (ES) cells and a site-specific recombination system (*loxP*/Cre), we have induced a deficiency^{5,6} that eliminates the products of the *Hoxd-13*, *Hoxd-12* and *Hoxd-11* genes simultaneously. A *Hoxd-11/lacZ* reporter gene replaced the deleted region in order to monitor the effect of this triple inactivation at the cellular level. Mice homozygous for this deficiency showed small digit primordia, a disorganized cartilage pattern and impaired skeletal mass. These alterations are similar to the defects seen in a human synpolydactyly^{7,8}, suggesting that this syndrome, which is associated with a subtle mutation in *HOXD13* (ref. 8), may involve the loss of function of several *Hoxd* genes. These results indicate the existence of a functional hierarchy among these genes and provide us with an animal model to study human digit malformations.

Vertebrate *Hox* genes are essential for the proper embryonic development of structures along the trunk and limb axes (for example, see ref. 9). In order to assess both the function and

regulation of posterior *Hoxd* genes in the ontogeny of digits, we generated a triple loss of function involving *Hoxd-13*, *Hoxd-12* and *Hoxd-11*. We used an embryonic stem (ES) cell line carrying an ectopic *Hoxd-11/lacZ* transgene located between the resident *Hoxd-13* and *Evx-2* genes and flanked at the 3' end by a unique *loxP* site¹⁰ (Fig. 1a; *Targ*). A *loxPneo* cassette was recombined on the same chromosome, downstream of resident *Hoxd-11* (Fig. 1b, c), so that a targeted deficiency could be induced after treatment with the Cre recombinase^{5,6} (Fig. 1d; *Del*). This deletion removed *Hoxd-11*, *Hoxd-12* and *Hoxd-13*, but brought the ectopic *Hoxd-11/lacZ* transgene back to the original *Hoxd-11* locus (Fig. 1d, e). Mice homozygous for this deficiency thus lacked *Hoxd-11*, *Hoxd-12* and *Hoxd-13* loci. The effect of the deletion was monitored by both the expression of the *Hoxd-11/lacZ* reporter, by β -galactosidase (β -gal) staining, and the limb phenotype.

We first compared *Hoxd-11/lacZ* expression in the predeleted targeted (*Targ*) with that in deleted (*Del*) chromosome (Fig. 1a, d). Although expression in digits was observed in both cases, the deficiency was accompanied by striking changes at early developmental stages. In *Del* forelimbs, reporter activation was observed as the bud emerged from the flank (Fig. 2a, b, left). In contrast, in the *Targ* configuration, β -gal staining appeared one day later (Fig. 2a, b, right). The onset and expression dynamics of the two different loci closely mimicked the endogenous *Hoxd-11* (ref. 11) and *Hoxd-13* (ref. 1), respectively, thus showing that expression of the same reporter gene changed following its location in the complex. Furthermore, it demonstrated that the deleted *HoxD* region was dispensable for expression in digit primordia. We analysed the expression of the two genes located on either sides of the deficiency: *Hoxd-10* and *Evx-2*, and found no significant

FIG. 1 Targeted deletion in the *HoxD* complex. a, 5' Region of the complex. This chromosome (*Targ*) has a copy of *Hoxd-11* with *lacZ* reporter sequences, resulting from previous gene transposition experiments¹⁰ (see brackets on the top). One *loxP* site (arrowhead) is left upstream of *Hoxd-13*. b, A *loxP*-PGKneo-*loxP* cassette was recombined 3' to the *Hoxd-11* locus of this *Targ* chromosome. c, The resulting chromosome with three *loxP* sites. d, Treatment of these cells with the Cre recombinase deleted ~30-kb of DNA encompassing *Hoxd-11*, *Hoxd-12* and *Hoxd-13*, meanwhile it repositioned the *Hoxd-11/lacZ* transgene at the resident *Hoxd-11* location. Consequently, the effective deficiency was ~23 kb, removing both *Hoxd-13* and *Hoxd-12* whereas *Hoxd-11* produced an inactive *Hoxd-11/lacZ* fusion protein. e, The various steps of this experiment were monitored by using a variety of different probes, as exemplified by an *EcoRI* Southern blot hybridized with probes A, B and C. For instance, the 4 kb *Hoxd-11* band, detected by probe A in heterozygotes (+/*del*) and homozygotes (*del/del*), but absent from (+/+), corresponded to the 3' region of the *Hoxd-11/lacZ* fusion gene. Probe B was internal to the



deletion and the presence of the 12 kb *Hoxd-10* *EcoRI* fragment in *del/del* verified the 3' border of the deletion (probe C).

differences (for example, *Hoxd-10*, Fig. 2c), suggesting that the deletion did not misregulate other genes in *cis*.

In homozygous *Del/Del* limb buds, the presumptive digit area was reduced in size, already at embryonic days 11 and 12 (E11 and E12) (see Fig. 2c) and digit defects as well as polydactyly were detected on the condensation pattern by E14 (not shown). Combined Alizarin and β -gal stainings of *Del/+* animals at birth showed the late and widespread expression of *Hoxd-11* in digits (Fig. 3a, b) and demonstrated the expression of the fusion gene in proliferative cells of the growth plates (Fig. 3c, P), whereas it was switched off in quiescent flattened cells (Fig. 3c, Q) and in hypertrophic cartilage (Fig. 3c, H). In *Del/Del* littermates, cartilage remodelling was dramatically delayed (Fig. 3d). Only few cells had switched off *Hoxd-11/lacZ* (arrows), whereas the characteristic cellular columns of quiescent cells were rarely visible (Fig. 3c, d; arrowheads). Hypertrophic cartilage cells were missing. Interestingly, wherever ossification was delayed, reporter gene expression persisted. Therefore, a correlation was observed, at the cellular level, between the absence of several *Hoxd* gene functions and a noticeable delay before the expressing cells progress into the histogenic sequence.

At birth, a conspicuous reduction in the size of both the paws and digits was scored as well as polydactyly (Fig. 4a, b). Cartilage staining confirmed the presence of 6 or 7 digit primordia (Fig. 4c, d). A few abnormally small and haphazardly located ossification centres appeared by the fifth postnatal day in the forepaws and, subsequently, in hindpaws. Syndactyly (digit fusions) was never

detected until this stage, indicating that it developed as a secondary fusion of developmentally retarded cartilage models (for example, Fig. 4c, digits III and IV). Adult skeletons revealed haplo-insufficient digit defects and recessive paw alterations. Although heterozygotes displayed a reduction of digits II and V, the absence of *Hoxd-11* to *Hoxd-13* function led to drastic size reductions (ectrodactyly) in distal elements, accompanied by bone fusions in the paws (Fig. 4e, f; *Del/Del*). Strongest defects were seen in digits, where polydactyly, fusions and webbing between digit vestiges were scored in all homozygotes. Secondary fusions between proximal digit elements produced Y-like digits, or synpolydactyly (Fig. 4f, arrows). Simple *Hoxd-13* mutant mice showed a much milder phenotype in digits^{1,3}, whereas mice mutated for either *Hoxd-12* (refs 3, 12) or *Hoxd-11* (refs 13, 14) alone were virtually normal. Yet the combined loss of functions generated very severe alterations. This illustrated the functional cooperation between these genes with, however, a prevalent role for *Hoxd-13*, as removing *Hoxd-12* or *Hoxd-11* functions had detectable effects only in absence of *Hoxd-13*.

These malformations phenocopied those reported in human patients with synpolydactyly, a dominant congenital disorder for which several pedigrees were linked to the *Hoxd* complex^{8,15}. Recently, this syndrome was associated with a short amplification of a polyalanine stretch within the first exon of *Hoxd-13* (ref. 8). A semi-dominant phenotype was observed and homozygous patients^{7,8} were considerably more affected than mice lacking all *Hoxd-13* function. Because our *Del/Del* mice phenocopied this syndrome, we believe that this congenital malformation is caused by the functional neutralization of several *HOX* proteins. During limb development, wild-type *HOXD-13* appears to be prevalent over other *HOXD* products¹⁰. A plausible molecular explanation is that the mutated human protein is functionally inactive while keeping all of its DNA binding properties. The functional prevalence of *HOXD13* (ref. 16) may thus suppress the functions of other *HOXD* and *HOXA* proteins normally involved in digit morphogenesis, for instance by binding site occupancy, leading to alterations more severe than those due to *Hoxd-13* inactivation alone. In this view, the human mutation would generate a dominant-negative version of *HOXD-13*. A related mechanism

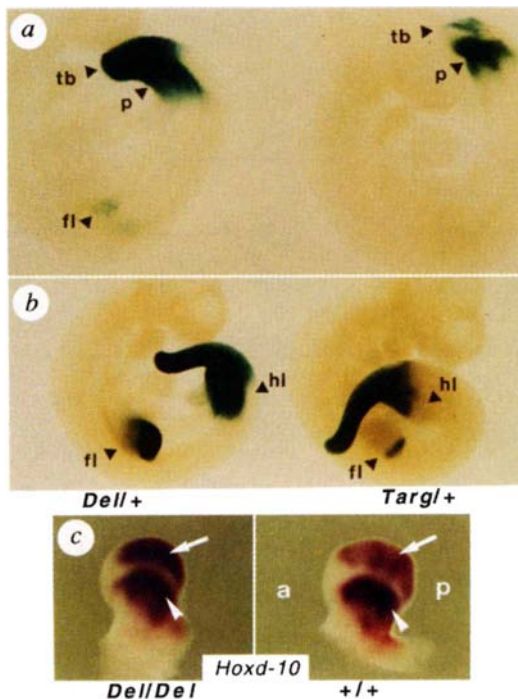


FIG. 2 Effect of the deletion on reporter gene expression. a, b, Expression of the *Hoxd-11/lacZ* reporter gene in littermates with (*Del/+*, left) or without (*Targ/+*; right) the deficiency. a, E9.5 embryos. Expression appeared in the forelimb mesoderm (fl) of *Del/+* mice whereas control limbs (*Targ/+*) are negative. In mutant mice, activation was first observed at E9, in the postero-ventral mesoderm, around the hindgut pocket and the future proctocaeal region (p), significantly earlier and about four metamers more anterior when compared to control mice; tb, tailbud. b, E10 embryos. *Del/+* mice had a more extensive expression domain in limbs at E10 and in subsequent stages, resembling the endogenous *Hoxd-11* pattern, whereas non-deleted animals expressed the transgene in limbs like the resident *Hoxd-13* gene. c, Expression of *Hoxd-10* in *Del/Del* and wild-type day 12 forelimbs. Both proximal (arrowhead) and distal (arrow) domains are maintained after deletion; a, anterior; p, posterior.

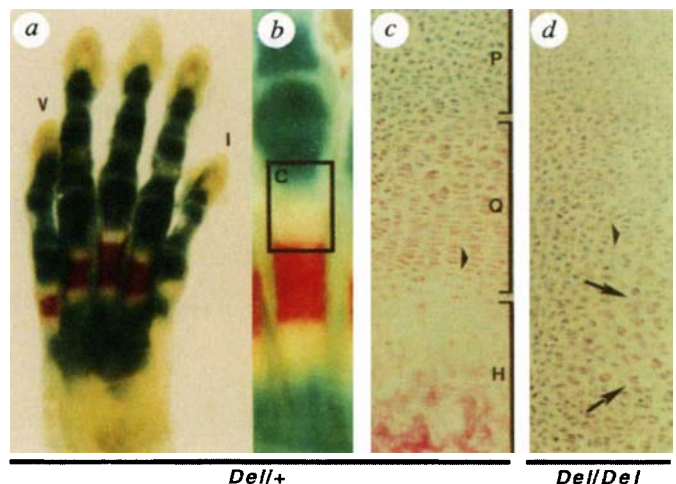


FIG. 3 Newborn *Del/+* hindpaw stained for both Alizarin red and β -gal activity. a, b, Cartilage models stained blue whereas a downregulation was observed before ossification occurred such that an unstained interface appeared on either side of Alizarin-positive segments. c, Histological section corresponding to the rectangle in b showing the blue-stained proliferative cells (P) and red-stained quiescent cells (Q); H, hypertrophic cartilage. d, A corresponding section from a *Del/Del* digit (same plane, same age). The zone of the proliferative and blue reporter-expressing cells is enlarged. Few columns of quiescent cells appear (arrows). Hypertrophic cartilage cells and mineralization are absent.

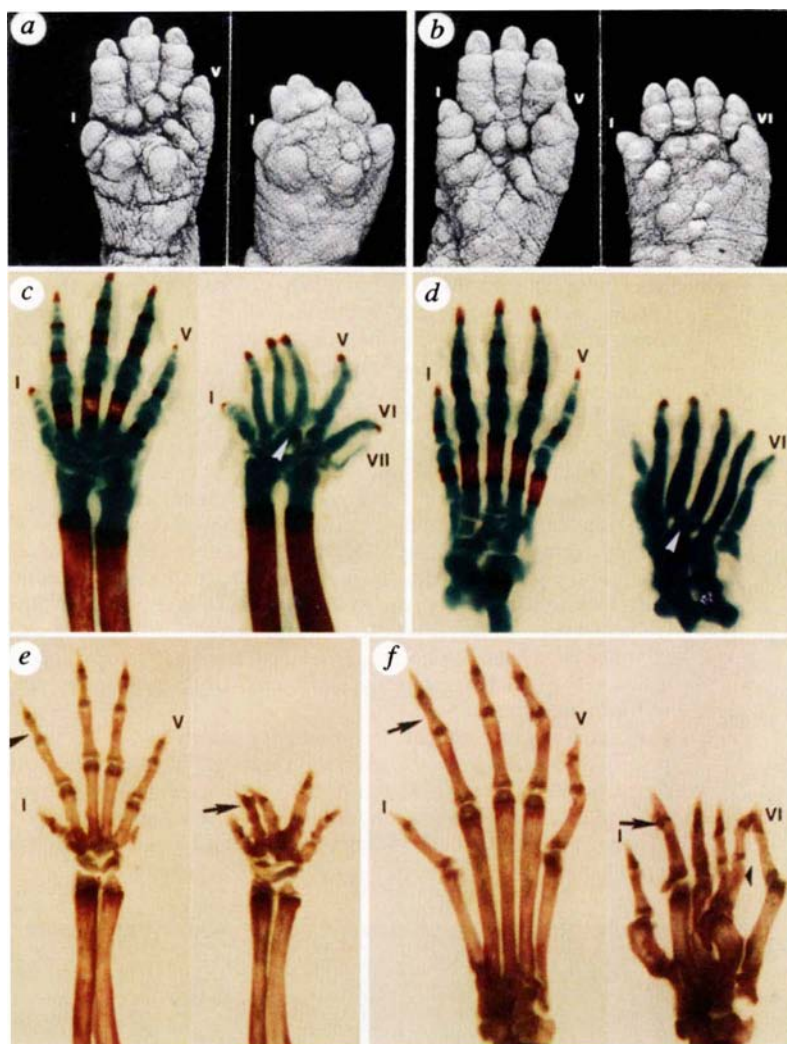


FIG. 4 Inactivation of *Hoxd-11*, *Hoxd-12* and *Hoxd-13* phenocopies human synpolydactyly. Scanning EM of *Del/Del* and *+/+* newborn forepaws (a) and hindpaws (b). Both ectrodactyly and polydactyly were observed. c, d, Skeletal preparations of the specimen in a and b, showing six or seven individualized digits, though some condensations articulate to the same carpal or tarsal bone (c, d; arrowheads). At birth, segmentation was incomplete and ossification centres absent. e, f, Adult forelimb (e) and hindlimb (f). Carpal bones were reduced, malformed and partially fused and phalanges were reduced in size and number in all but digit I (for example, arrowhead). Digit I-II-III as well as IV and V were joined in the forepaws and fusion of two condensations led to a thick digit IV. f, Additional digits were intercalated or fused, either to digit III or IV, leading to synpolydactyly (arrowhead). There were invariably six defective and partially fused digits. More digit vestiges were seen in newborns as compared to adults, indicating that the final digit number arose through late secondary fusions.

was recently proposed for RTH (ref. 17), another dominant human syndrome involving transcription factors.

In conclusion, the deletion of three posterior *Hoxd* genes clarified several aspects of the regulation and function of *Hox* genes in digits. First, it unambiguously confirmed that temporal activation of a given *Hoxd* gene in limbs can vary depending upon its position in the complex. Second, it demonstrated that these genes control initially the allocation and growth of digit prechondrogenic condensations and, subsequently, the growth and ossification sequence of the cartilage models. Finally, this deficiency provides us with the first animal model for a human digit disorder and suggests a genetic and mechanistic explanation for the aetiology and pathogenesis of human synpolydactyly. □

Methods

A *loxP*-PGKneo-*loxP* selection cassette was introduced into the unique *EcoRI* site present between *Hoxd-11* and *Hoxd-10* and further electroporated in ES cells that already contained the *Hoxd-11/lacZ* fusion gene (TgH[d-11/lac]Ge in ref. 10) between *Hoxd-13* and *Evx-2*, flanked at the 3' by a *loxP* site (*Targ* chromosome on Fig. 1). Two independent re-targeted clones were isolated and introduced into the germ line of chimaeric male mice. *loxP* sites were oriented in the same direction so that electroporation of a plasmid expressing the Cre recombinase in these cells resulted in the loss of the genomic region flanked by *loxP* sites. The deletion was monitored by the loss of both an *EcoRI* genomic fragment and the *neo* gene. The deficiency was induced both *in vitro* and *in vivo*. In the latter case, a cytomegalovirus promoter-driven Cre recombinase expressing transgenic mouse was used. The expression patterns of the *Hoxd-11/lacZ* reporter gene as well as the phenotypes of mutant mice derived from one *in vivo* and two *in vitro* induced deletions were identical. Production of staged embryos and detection of β -gal activity was performed as described in ref. 10. Newborn limbs were dissected, skinned, fixed for 4 h in 4% paraformaldehyde, rinsed and incubated for 6 days in standard X-gal cocktail at room temperature. After staining, they were rinsed three times and incubated in PBS complemented with 0.03% Alizarin red S for an additional 6 days. Such preparations show the expression domain of the *Hoxd-11/lacZ* fusion gene, present in the *Del* locus, (β -gal activity) in blue and the progression of ossification, in red. The specimens were further equilibrated in 20% sucrose, sectioned with a cryostat and counterstained with eosin. For skeletal staining, animals were killed as newborns or at 5 weeks of age (young adults) and stained according to standard Alcian blue/Alizarin red S, or Alizarin alone, staining protocol. In heterozygotes, the second phalanges were reduced in size and defective in ossification by visual inspection. Genotypes were confirmed by Southern blot analysis of genomic DNA isolated from kidneys of killed animals or from tail biopsies. Whole-mount *in situ* hybridizations were carried out following a standard procedure.

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wingless refines its own expression domain on the *Drosophila* wing margin

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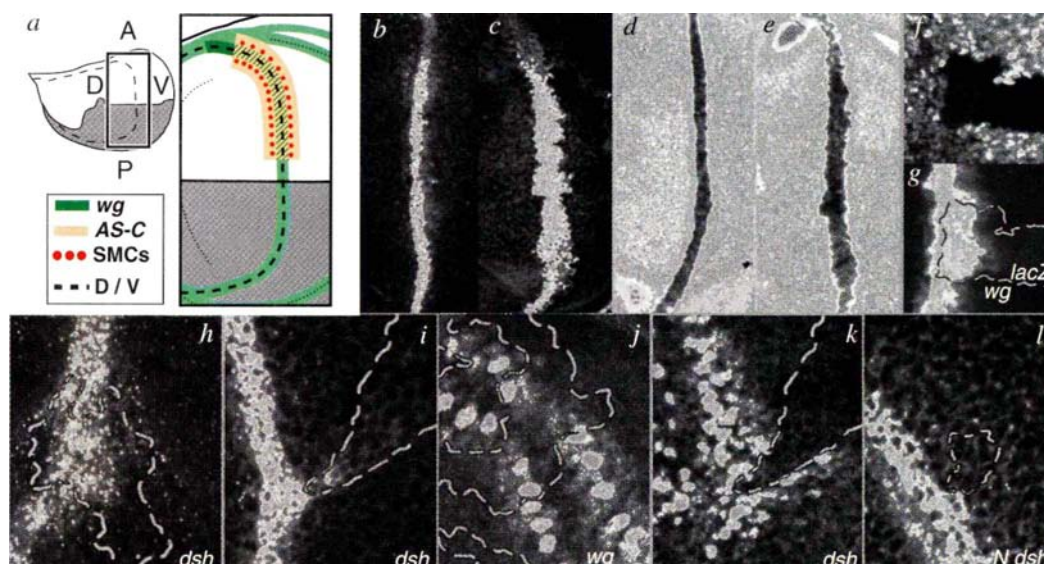
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THE imaginal discs of *Drosophila*, which give rise to the adult appendages, are patterned during a period of intense cell proliferation. The specification of differing regions occurs in some cases by subdividing the disc epithelium into lineage compartments¹. However, in most cases precise boundaries are formed between different cell types without early compartmentalization². One such boundary occurs between the *wingless* (*wg*)-expressing cells of the wing margin and the adjacent proneural cells, which give rise to margin sensory bristles. Here we show that this boundary arises in part by a mechanism of 'self-refinement', by which *wingless* protein (Wg) represses *wg* expression in adjacent cells. Cells unable to receive the Wg signal do not resolve the boundary between *wg*-expressing and proneural cells.

The wing imaginal disc is set aside during embryogenesis as an anlage of 20–40 cells, which by the end of three instars of larval life has proliferated to form a single-layered epithelium of roughly 50,000 cells^{1,3}. Midway through the second instar, the disc becomes subdivided into dorsal and ventral lineage compartments; in the prospective wing blade, the cells surrounding the dorsoventral boundary form the wing margin and express a number of margin-specific genes. In early third-instar wing discs (48 h before pupariation), the secreted *Wg* growth factor is expressed at low levels throughout the prospective wing blade. Beginning at mid-third instar (24 h before pupariation), this pattern refines, becoming expressed (Fig. 1a) at high levels along the prospective wing margin in a stripe 3–6 cells wide just to either side of the dorsoventral boundary (Fig. 1b, d). Shortly thereafter, the proneural transcription factors genes *achaete* (*ac*) and *scute* (*sc*) are expressed in a broad stripe 10–12 cells wide along the anterior margin. Proneural expression is reduced within, but is high adjacent to, the *wg*-expressing cells. The sensory mother cells (SMCs) that give rise to the chemosensory margin bristles arise within this proneural region, immediately adjacent to the *wg*-expressing cells (the SMCs are first detected by heightened proneural gene expression; Fig. 1j, k). Other expression patterns also obey this boundary: the *wg*-expressing cells express *cut*⁴, the *vestigial* intron-2-*LacZ* construct⁵, and certain *Enhancer of split* complex members^{6–8}, and are flanked by regions expressing high levels of the Notch ligand genes *Delta* and *Serrate* (not shown) and reduced amounts of *Notch*⁹.

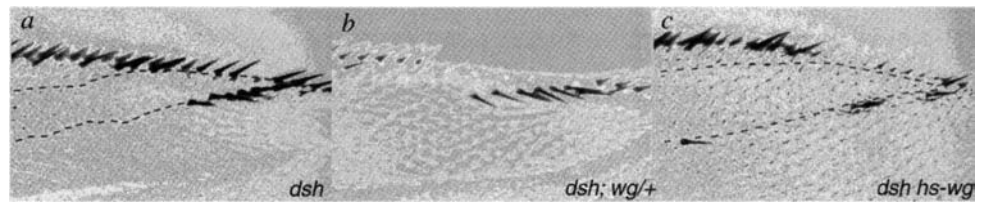
Reductions or removal of Wg activity resulted in a failure to resolve this boundary properly. The *wg^{ΔL}* allele is temperature sensitive; at the restrictive temperature it acts as a strong hypomorph, but produces an immunologically detectable product¹⁰. When reared at restrictive temperatures for 12 h, late third instar

FIG. 1 a–i, Margin *wg* expression after loss of *wg* or *dsh* functions. a, Diagram of late third instar wing disc. Box outlines margin region shown in the remaining figures, with axes and patterns of gene expression as marked (see text for details). AS-C, *achaete-scute* complex expression; D/V, dorsoventral. b–e, *wg^{ΔL}* (*wg^{ΔL}/wg^{cx4}*) margins, stained for Wg protein (b, c) or messenger RNA (d, e) expression. b, d, At permissive temperature, protein and mRNA expression was normal. c, e, After a 12 h shift to restrictive temperature, protein expression expanded to a region twice the normal width (protein: 55/57 discs; mRNA: 17/19 discs). f, g, Hypomorphic *wg^{LacZ}* clone on the margin, shown by the absence of anti-Myc staining (f; in g and subsequent panels clones are marked by a dotted outline and *). Clones caused expansion of *wg-LacZ* expression (anti-β-gal; 72/97, none showed loss). h, i, *dsh* clones that intersected (h) or sat immediately adjacent to (i) the *wg* stripe elevated anti-Wg-staining cell-autonomously (*dsh⁷⁵*: 15/17; *dsh^{v26}*: 12/12). Similar effects were observed in anterior, posterior, dorsal and ventral clones. In some cases, it seemed that the normal *wg* stripe expanded or distorted to meet the ectopic anti-Wg staining within clones away from the margin (see i; 7 clones). It is unclear whether ectopic *wg* expression is being induced in wild-type cells between the clone and the margin, or if cells at the margin are distorting or rearranging near the clone. j, k, Anti-Scute or Achaete staining in *wg^{ΔL}* and *dsh^{ΔL}* clones. j, *wg^{cx4}* clones that intersected the *wg* stripe showed non-autonomous loss of Sc (18/22 large clones), as did 5/7 *wg^{LacZ}* clones (not shown). Sc was expressed at wild-type levels in *wg* mutant cells that were 1–2 cell diameters from the



clone boundary, presumably in response to Wg secreted by wild-type cells. Most small clones, where all cells were close to the boundary, showed normal Sc levels. k, *dsh^{ΔL}* clones within the margin proneural region showed cell-autonomous loss of sc expression (*dsh⁷⁵*: 9/9; *dsh^{v26}*: 15/15). *dsh* clones that intersected the *wg* stripe, and produced ectopic *wg*, also generated ectopic sc expression outside the normal proneural region in *dsh^{ΔL}* cells near the clone boundary (arrow; note *wg* expression in same clone in i). l, Margin *wg* expression after loss of *Notch* (N) and *dsh*. *Notch^{ΔL} dsh^{ΔL}* clones that lay adjacent to (l) or intersected the *wg* stripe (not shown) showed cell-autonomous loss of anti-Wg staining without expansion of *wg* expression (29/29 clones). *Notch^{ΔL} dsh^{ΔL}* clones in the anterior also lose anti-Sc staining (E.J.R., C.A.M., M. Halevy and S.S.B., manuscript in preparation).

FIG. 2 *dsh*⁻ clones induced ectopic bristles off the wing margin in a *wg*-dosage-dependent fashion. **a**, *dsh*⁻ clone in adult wing, marked with *y* and *f*^{26a} (dotted outline), extending from the margin into the interior of the wing blade. The average distance from farthest bristle to margin was 4.5 cells (± 1.0 , range 3–7, *n* = 23). **b**, *dsh* clone induced in a *wg*⁶²² heterozygote. The average distance for all clones observed was 2.71 (± 0.73 , range 2–4, *n* = 14). **c**, *dsh* mutant clone induced in the presence of heat-shock promoter-*wg*. The average distance for all clones observed was 10 cells (1.6, range 8–12, *n* = 5). *hs-wg* produces insufficient activity to generate ectopic bristles in a wild-type background (not shown). The clones in **b** and **c** are not marked with *f*^{26a}, but are identified by the cell-autonomous tissue polarity defect caused by *dsh*¹⁴ (results not shown). Unmarked *dsh*⁻



and *Notch*⁻ *dsh*⁻ clones were identified in adult wings by the absence of normal margin bristles^{6,20}. 22/30 *dsh* clones were accompanied by ectopic bristles distant from the margin, as compared with 5/40 *Notch* *dsh* clones. The bristles near *Notch* *dsh* clones were only rarely as contiguously arrayed or as distant from the margin as those near *dsh* clones. Regulation within the margin proneural regions may account for these occasional bristles (data not shown).

wg^{FL}/*wg*^{null} discs (0–8 h before pupariation) showed widened margin expression of *wg* product (Fig. 1c) and mRNA (Fig. 1e) in a stripe of roughly twice its normal width. We obtained similar results using mosaic analysis. An enhancer-trap insertion into *wg*, *wg*^{LacZ}, is a strong hypomorph¹¹. Most homozygous *wg*^{LacZ} clones within the normal *wg*-expressing region induced expansion of *wg*-*LacZ* expression in flanking cells outside the normal region (Fig. 1f,g). Thus, cells immediately outside the normal stripe of *wg* expression are capable of expressing *wg* at late third instar, and *wg* expression within the normal *wg* stripe directly or indirectly represses *wg* expression in those adjacent cells.

To test whether this interaction was direct, we used mosaic analysis to render the adjacent cells incapable of receiving the Wg signal. The ubiquitously expressed cytoplasmic protein encoded by *dsh* is required for the reception of Wg signals^{12–14}; the evidence suggests that the *dsh* protein, Dsh, is activated by the Wg signal^{15,16}. The *wg* gene was ectopically expressed in *dsh*⁻ clones located near the normal *wg* stripe (Fig. 1h, i), as would be expected if the interaction was direct. Expression of *wg* expanded as many as six cells from the normal *wg* boundary. Thus, reception of Wg signalling is required to repress *wg* expression in a broad region of competence near the normal margin. This novel self-refinement function of Wg cannot on its own account for the normal retention of *wg* expression along the margin, which must result from some preexisting bias (see below). Nonetheless, Wg plays a critical role in localizing the boundary between *wg*-expressing and non-expressing cells.

Self-refinement is one of two identified signalling functions of margin Wg. Previous work has shown that Wg signalling is necessary and sufficient for proneural gene expression and the subsequent formation of margin bristles in cells flanking the *wg*-expressing stripe^{11,12,16–21} (Fig. 1j, k). Thus, removing the ability to respond to the Wg signal by generating *dsh*⁻ clones results in the

loss of proneural gene expression (Fig. 1k) and bristles in the adult^{16,20} (Fig. 2a). One might also predict that the ectopic *wg* expressed within the clone might be able to induce ectopic proneural gene expression in wild-type cells surrounding the clone, leading to the formation of margin bristles abnormally distant from the margin. This phenotype has indeed been reported in adult wings²⁰ (Fig. 2a), and we observed clearly ectopic proneural gene expression surrounding *dsh*⁻ clones (Fig. 1k). It is likely that the ectopically expressed *wg* plays an important role in this phenotype. The formation of ectopic bristles was sensitive to the expression of *wg*, as ectopic bristles were found a shorter distance from the margin in a *wg*⁻/*+* background (Fig. 2b), and further from the margin after *wg* overexpression using heat-shock promoter-*wg* (Fig. 2c). Moreover, those manipulations that did not lead to ectopic *wg* expression, such as generating *Notch*⁻ *dsh*⁻ double-mutant clones (see below), only rarely induced the formation of ectopic bristles (Fig. 2 legend).

One possible way that Wg self-refinement might act is by repressing the Notch signalling pathway. The *Notch* gene is required for normal *wg* expression along the margin^{3,21}, and ectopic expression of Notch ligands or raised Notch activity can induce ectopic *wg* expression and margin-like development^{8,21–24}. It has therefore been suggested that heightened Notch activity along the dorsoventral boundary is responsible for the localized expression of boundary-specific genes, including *wg*. Moreover, recent evidence indicates that Dsh-mediated Wg signalling can inhibit Notch activity¹⁶.

Although we cannot rule out other mechanisms, our data are consistent with the hypothesis that Wg inhibits Notch. If Wg signalling inhibited margin Notch activity, any increase in the Wg signal should reduce and narrow the domain of the Notch-mediated *wg* expression along the margin, as does lowering Notch activity³. As predicted, the increase in Wg signalling induced by

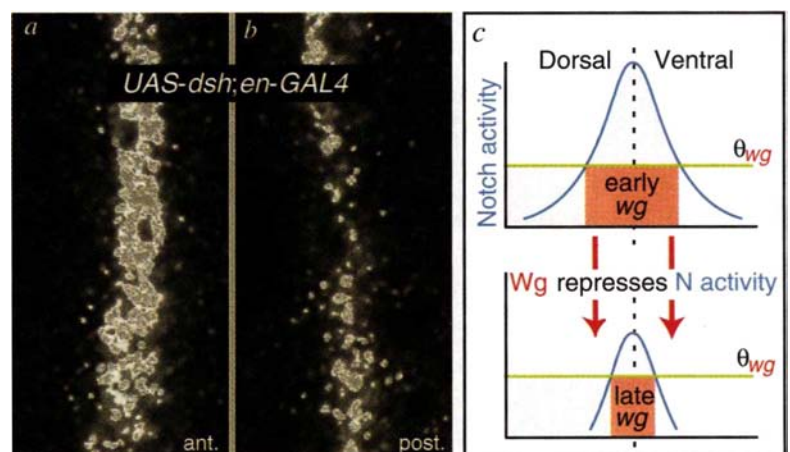


FIG. 3 Interactions between *Notch* and *dsh*. **a**, **b**, *dsh* overexpression in the posterior compartment, using *UAS-dsh* and *en-GAL4*, caused narrowing and occasional loss of margin anti-*wg* staining (**b**) when compared with anterior (**a**). *Dsh* levels, as identified using anti-Dsh, were visibly higher in the posterior compartment (not shown). Both panels are from the same imaginal disc. **c**, Model of Wg self-refinement. Notch signalling activity is highest at the dorsoventral boundary, and levels above the threshold (θ_{wg}) initially trigger broad *wg* expression (above). Wg represses Notch (N) activity; *wg* expression is maintained only in cells nearest the dorsoventral boundary (below).

the overexpression of Dsh¹⁶ in the posterior compartment had exactly this phenotype (Fig. 3a, b). A further prediction of this model is that the ectopic expression of wg observed in *dsh*⁻ clones should be reversed by the simultaneous loss of *Notch*; indeed, ectopic wg expression was not observed in *Notch*⁻ *dsh*⁻ clones (Fig. 1f). The mechanism by which Wg inhibits Notch activity is not known, but it has been suggested that this inhibition is mediated by the binding of Dsh to Notch¹⁶. One prediction of the Dsh–Notch-binding model is that removal of Wg signalling components downstream of Dsh should not affect Notch activity. Our evidence suggests that *Armadillo*, which like *dsh* is required for normal Wg signalling but which acts genetically downstream of *dsh*^{25,26}, is not required for Wg self-refinement (E.J.R., C.A.M., M. Halevy and S.S.B., manuscript in preparation).

That a narrow region of wg expression is normally retained along the margin, even after self-refinement, indicates that these cells are in some manner less sensitive to Wg signalling than cells more distant from the margin. This difference in sensitivity could be explained in two ways. First, some unknown factor specific to the dorsoventral boundary may render boundary cells less sensitive to Wg signalling. Recent evidence suggests that there are as yet uncharacterized signals organized around the dorsoventral boundary²⁷, and these could be responsible for localized biases in cell behaviour. A simpler hypothesis is that Notch activity at the dorsoventral boundary is initially higher and thus remains above levels required for wg expression (Fig. 3c). In support of this idea, it should be noted that both *Enhancer of Split* complex members and the *vestigial* second intron enhancer are expressed specifically along the margin, and that this expression depends upon the presence of Su(H) binding sites within their enhancers^{6,7,27}; the Su(H) transcription factor is thought to mediate Notch signalling.

The self-refinement function of Wg may have parallels in other situations, including the vertebrate hindbrain. As in the wing margin, the boundary-specific domains of *Wnt-1* expression are initially sloppy, but become refined later in development; moreover, in *Wnt-1*^{sw} mutant mice many of the domains of *Wnt-1* hindbrain expression seem to be expanded when compared with the wild type²⁸. □

Methods

wg^{ts} larvae were from *wg^{ts}/ln(2LR) Glu Bc¹ X wg^{ts}/ln(2LR) Glu Bc¹*; permissive and restrictive temperatures were 16.5 °C and 30 °C, respectively. Shifted discs and unshifted controls were marked and labelled in the same well. Antibody labelling was as described previously³, using rabbit or rat (1/1000) anti-Wg (provided by R. Nusse), 1/1000 rabbit anti-Scute or 1/25 mouse anti-Ac (both provided by G. Panganiban), 1/400 rabbit anti-Dsh¹⁵ (provided by R. Nusse), anti-Myc supernatant, and/or anti-β-galactosidase. *In situ* hybridization was as described previously²⁹ with dig-labelled wg complementary DNA (provided by F. M. Hoffmann). Clones were generated using the *FLP/FRT* system as described previously³ with the following crosses: *wg^{lacZ} FRT^{40A}/CyO X y w FLP1; πM^{21C} πM^{36F} FRT^{40A}* (provided by A. Penton). *svb^{VP170} dsh^{Y26} FRT¹⁰¹/FM7* or *y w dsh^{Y26} FRT¹⁰¹/FM7* or *N¹⁰⁸¹ svb^{VP170} dsh^{Y26} FRT¹⁰¹/FM7 X πM^{5A} FRT¹⁰¹; FLP3, Sb/TM6, Tb. y w dsh^{Y26} FRT⁹⁻²/FM7 X ovo^{D1} FRT⁹⁻²; FLP³⁸/FLP³⁸. y w dsh^{Y26} FRT¹⁰¹/FM7; *wg^{G22}/Glu X ovo^{D1} FRT¹⁰¹; FLP³⁸/FLP³⁸. y w dsh^{Y26} FRT¹⁰¹/FM7; *hs-wg/+ X ovo^{D1} FRT¹⁰¹; FLP³⁸/FLP³⁸. dsh^{Y26}* and *wg^{G22}* are protein nulls^{15,30}, whereas the sole phenotype of *ovo^{D1}* is female sterility. Larvae observed at late third instar (Fig. 1) were heat-shocked during second instar, and those reared to adulthood (Fig. 2) were heat-shocked during third instar. All were reared at 25 °C. Dsh overexpression was induced using the *GAL4/UAS* system as described previously¹⁹, with *UAS-dsh X en-GAL4*. These larvae were reared at 22 °C.**

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Shared neural control of attentional shifts and eye movements

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WE are able to move visual attention away from the direction of gaze, fixating on one object while attending to something else at a different location, within the region of peripheral vision. It has been widely assumed that the attentional neural systems are separate from the motor systems, but some studies challenge this idea^{1–5}. It has now been suggested that the attentional system is part of the premotor processing in the brain⁶. This model proposes that attentional processes evolved as part of the motor systems, with isolated attentional shifts representing an artificial separation of a natural linkage. Here we test how attentional shifts might be linked to the preparations for making saccadic eye movements. We studied the superior colliculus in monkeys as they shifted their attention during different tasks, and found that each attentional shift is associated with eye-movement preparation.

Attention can be moved under voluntary or involuntary control^{7–9}. A technique developed to study experimentally the dynamics of visual attention involves presentation of a cue, which indicates the target position before onset of that target⁸. This improves accuracy of target detection and decreases the time needed to detect or identify the target. Researchers refer to voluntary control of attention as endogenous and study it with symbolic cues. The involuntary control has been called exogenous (or reflexive), and is studied with cues directly priming a location.

Previous reports have proposed that the superior colliculus participates in shifting attention exogenously¹⁰. Patients suffering from tectal lesions often have deficits in the ability to shift attention¹¹. The superior colliculus is also important in the generation of eye movements^{12,13}. We have explored the relationship

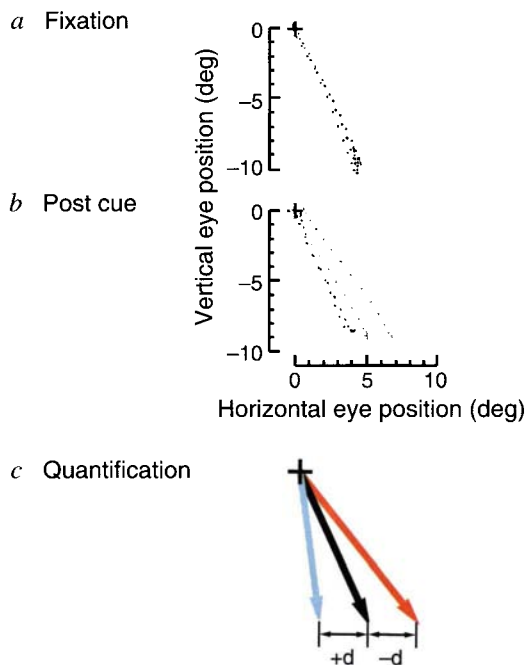


FIG. 1 *a*, Three fixed-vector saccades evoked during periods of fixation. *b*, Modified saccades after the cue onset. X-Y plots of the monkey's eye position; 2 ms between dots. Monkey fixated a central stimulus represented by +. The site of a burst cell, 1.7 mm from the collicular surface, was stimulated. In *b*, after the monkey maintained fixation for at least 1 s a cue appeared indicating the target location (12° to the left or right of the fixation point). At various random times after the cue onset, the superior colliculus was stimulated (five trials). The direction of the saccade evoked by collicular stimulation was different from that of the fixed vector saccade and shifted toward the cued location. Red traces were evoked after the right cue, blue were after the left cue, and black were evoked at the instant of the cue onset. *c*, Scheme showing how we analysed these data. Amount of shift of the evoked saccade was calculated as a distance (*d*) between end points of the averaged fixed-vector saccade and the saccade elicited after the cue onset. Positive values were arbitrarily assigned to modifications to the left.

We studied six colliculi in three macaque monkeys (*Macaca mulatta*). Our data include a total of 23 penetrations into the colliculi and 20 sites which we recorded from and stimulated. When the monkeys performed the peripheral-cue eye-movement task, targets that had been cued in the correct location (valid cue) were associated with faster eye-movement reaction times than those that were cued in the incorrect hemifield (invalid cue) (Table 1). Thus, each cue caused a shift in visual attention to its locus and facilitated saccadic eye movements to that location¹⁶. We next tested whether this shift of attention had any effect on the oculomotor systems. When the monkeys simply fixated on a target, repeated stimulation evoked uniform fixed-vector saccades (Fig. 1*a*). However, when we stimulated the superior colliculus after the onset of cues, there were shifts in the direction of the evoked saccades (Fig. 1*b*). We measured the distance between the end of a fixed-vector saccade and the end point of the saccadic eye movement evoked at various times after the cue onset (Fig. 1*c*)¹⁷.

For validly cued targets, the saccadic eye movements evoked by stimulation of the superior colliculus were deviated in the direc-

between attention and eye movements by using the stereotyped saccadic eye movements evoked by collicular stimulation^{12,14}. Our method was based on evidence that collicular stimulation applied during the preparation for a saccadic eye movement results in premature, imprecise saccades¹⁵. We analysed changes in the evoked saccades when the monkey moved its attention to the periphery before stimulation.

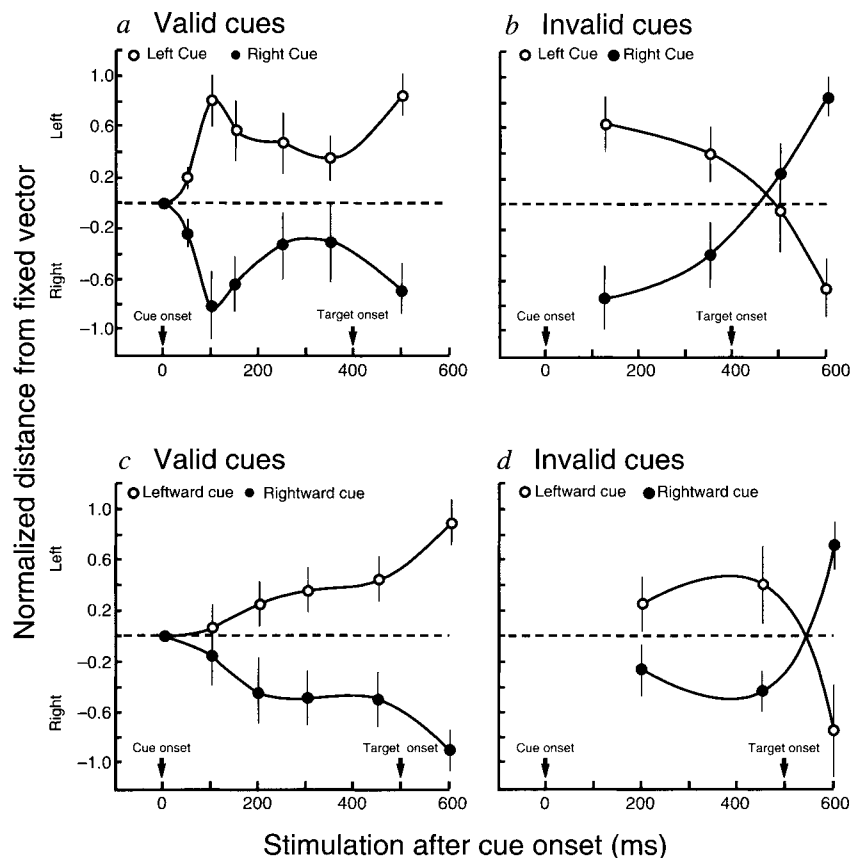


FIG. 2 Effects of peripheral cueing (*a*, *b*) and symbolic cueing (*c*, *d*) in eye-movement tasks on the shift of the evoked saccade. For all plots, the vertical axis is the normalized distance between the end points of the fixed-vector saccades and task-evoked saccades (see Fig. 1). Data from each experiment were normalized using the formula: $(d - d_0)/(d_{\max} - d_0)$, where $d_{\max}$ is the maximal shift of saccades evoked from that site and d_0 is for saccades evoked at cue onset. Vertical bars, s.d. The horizontal axis is the time of stimulation after cue onset for the one cue-target interval illustrated. The 80% valid (*a* and *c*) and 20% invalid (*b* and *d*) cueing trials, cue-target intervals, and sides were randomly interleaved. The curves were generated from spline fitting of the data. The data in *a* and *b* represent the average change for 10 different collicular sites; those in *c* and *d* come from 11 loci studied with symbolic cueing.

TABLE 1 Reaction times from target onset to saccade

SOA	Eye movement task						Manual task					
	Peripheral cues			Symbolic cues			Peripheral cues			Symbolic cues		
	100	300	500	100	300	500	100	300	500	100	300	500
Valid reaction times	249 ± 5	233 ± 11	267 ± 5	247 ± 6	211 ± 5	206 ± 5	420 ± 7	362 ± 8	352 ± 8	396 ± 7	382 ± 5	375 ± 6
Invalid reaction times	295 ± 17	315 ± 11	310 ± 19	283 ± 12	244 ± 10	211 ± 19	413 ± 8	403 ± 10	398 ± 8	406 ± 7	409 ± 6	407 ± 5
Validity effect	46*	82**	43*	36*	33**	5	-7	41**	46**	10	27**	32**

Behavioural data collected outside recording sessions, presented as means ± s.e.m.. SOA, stimulus onset asynchrony. Validity effect is the difference between the reaction time means of valid and invalid cue conditions. * $P \leq 0.05$, ** $P \leq 0.01$, significance of validity effect as calculated by the Wilcoxon test.

tion of the cue and target. Figure 2a shows the shift with the variable time of stimulation in relation to the onset of the cue. In contrast, when the cue appeared in one visual field, but the target appeared in the opposite field (invalid trials), the evoked eye movements were initially shifted towards the cued side. After the onset of the target, this shift began to reverse (Fig. 2b). As can be seen for all these data, the parameters that determine the degree of rotation are the time delay between the cue onset, target onset and moment of collicular stimulation. Whereas others have found that the motor preparation to make an eye movement can modify the electrically evoked saccade¹⁸, we show that the shift of attention also leads to modifications of the evoked saccades.

We also used a 'central symbolic cueing' task, in which the animals translated the colour of a foveal cue into the spatial location of the forthcoming target. Again we obtained appropriate effects of cueing on the reaction times for eye movements (Table 1). As can be seen in Fig. 2c and d, symbolic cueing altered the stimulation-evoked eye movements.

From these results, we cannot rule out the possibility that changes might be due to the preparation for making an eye movement. Therefore we trained one of the previously tested monkeys and a totally naive animal on new peripheral- and symbolic-cueing tasks. Here the monkeys had to maintain fixation

on the central stimulus throughout the trial and were trained to respond only with hand movements. Typical reaction-time data in Table 1 show attentional effects. These manual tasks produced similar deviations of the eye movements evoked from stimulation of the superior colliculus (Fig. 3). With peripheral cueing, there is strong and stable modification of the evoked movement, which reversed directions after invalid cues (Fig. 3a, b). The effects in the symbolic cueing task appear more gradually (Fig. 3c, d), just as the translation of the coloured signal must be more gradual than for a peripheral flashed cue. These data show that attentional shifts that are independent of eye movements to the targets still lead to modifications of the evoked saccades.

'Build-up' cells in the intermediate layers of the superior colliculus have progressive increases in activity during the preparation of saccadic eye movements¹⁹. Moreover, such cells can modulate with anticipation of an eye movement towards a specific spatial location^{19,20}. In the monkeys that performed the attention tasks with eye-movement responses, we found that build-up cells responded in a time-locked fashion when the peripheral cue appeared in the receptive field (Fig. 4a). Importantly, build-up cells responded to the symbolic cueing (Fig. 4b). The timing and level of activity were significantly changed with the timing of stimulation ($F = 3.654$, $P < 0.01$) and also differed from the peripheral cueing task. Even

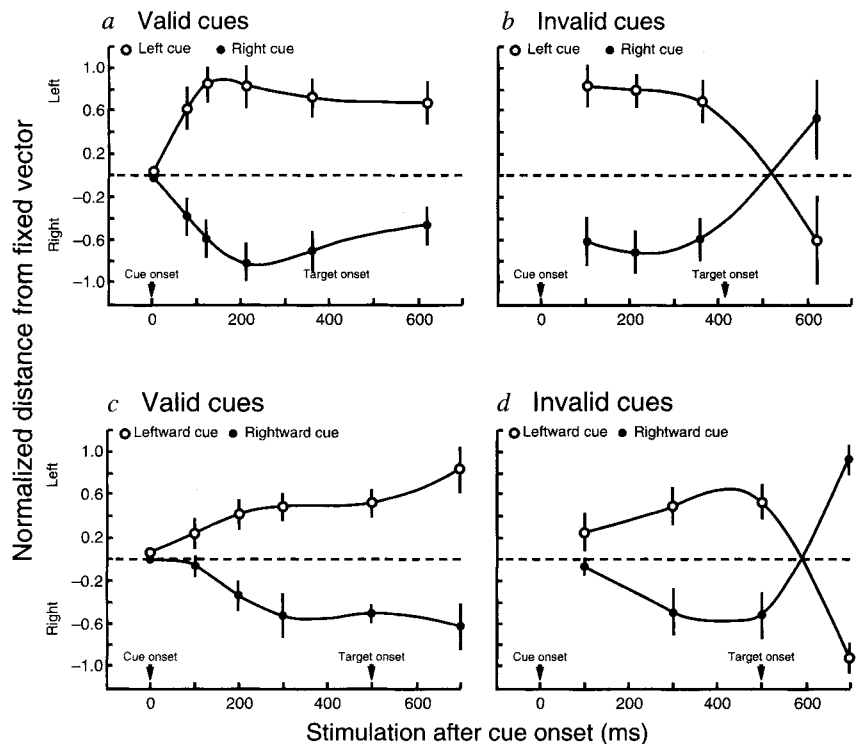
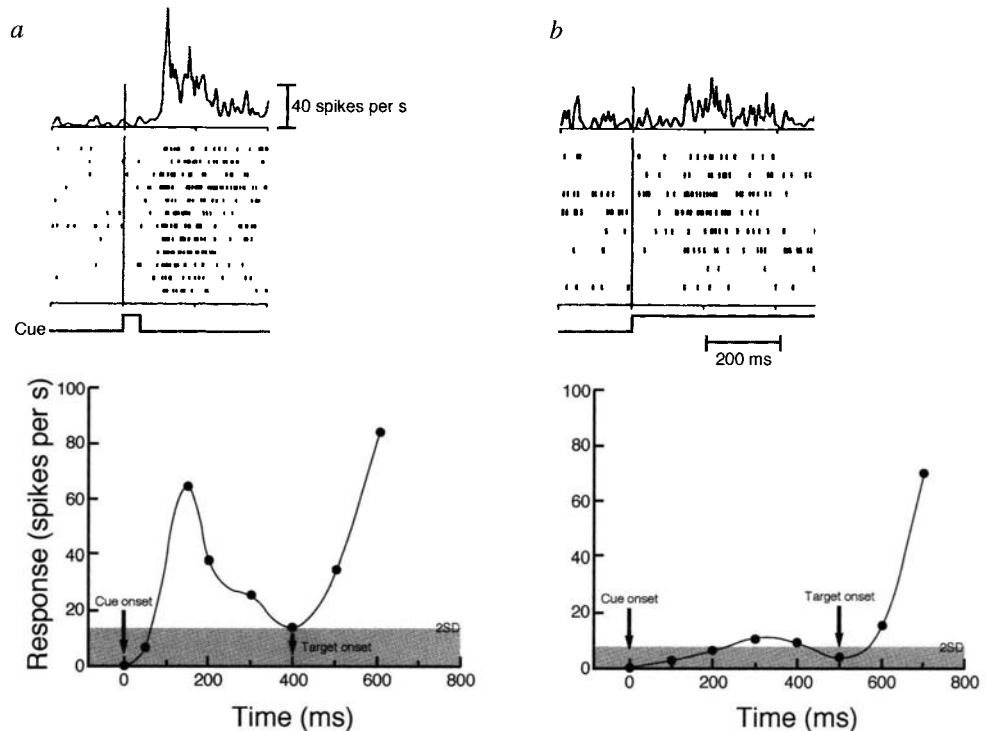


FIG. 3 Effects of peripheral cueing (a, b) and symbolic cueing (c, d) on the shift of the evoked saccade during a hand-response task. The format of the figure is as Fig. 2. The data in a and b represent the average of 8 different collicular sites, whereas those in c and d come from 7 loci.

FIG. 4 Activity of build-up neurons during peripheral (a) and symbolic (b) cueing tasks. Top panels: responses of the cells to the cue onset while the animal fixated a central spot of light. Each horizontal row represents one trial and each dot corresponds to a single spike. Spike-density curves are shown at the top. Vertical line represents cue onset. Bottom panels: cell activity was calculated as the number of spikes in 50- or 100-ms intervals and normalized against its baseline activity during simple fixation. The shading at the bottom of each graph represents two standard deviations from the mean background activity of the cell. Each data point on the curve shows averaged activity of 8 (a) and 6 build-up cells (b) at different times after the cue or target onset, indicated by arrows. Similar data were obtained at other cue-target intervals (not illustrated). Smooth lines are spline-approximation curves. Note a sharp increase in cell discharge after the target appearance, which is the pre-saccadic burst response.



when no stimulus appeared in the receptive field of a build-up cell, the central cue induced a build-up response. This means that the translation of the symbolic cue occurred within the saccadic system (possibly in parietal cortex), reached the superior colliculus, and was used to prepare a saccade.

Stimulation of the superior colliculi of monkeys during fixation leads to stereotyped eye movements. When cues (peripheral visual or foveal symbolic) shift attention, there is a consistent shift in the direction of the stimulation-evoked eye movement. This indicates that shifts of attention might be associated with the preparation to make an eye movement towards the attended location. Recordings from build-up neurons in the superior colliculus reveal responses to the attentional cueing and modulation with the intention to look at the specified location. These observations indicate that the build-up cells in the superior colliculus might be participating in the shift of attention as well as the preparation to make an eye movement, and they differ from the cells in the collicular superficial layers which are not modulated by endogenous attentional shifts¹⁰. However, the initiation of a saccade in such experiments can be under voluntary suppression which involves other neural centres^{5,21}. Our data are consistent with the hypothesis that shifts of attention, however evoked, are tightly coupled with the preparation to make oculomotor responses to the attended area.

The strength of the effects of peripheral cueing on stimulation-evoked saccades supports the idea that the superior colliculus is involved in reflexive attentional shifts. On the other hand, sym-

bolic cueing does require an analysis of the meaning of the sensory input (voluntary attention). It has been suggested that such analysis might be done in posterior parietal cortex²². Parietal neurons send strong projections to the superior colliculus^{23,24}, and the effects we report here may be mediated by parietal cortex. □

Methods

Techniques have been described previously^{17,25}. In the peripheral cueing task, the monkeys fixated a central spot, and at some variable time later, a large light was flashed briefly (16 or 50 ms) in one hemifield^{8,16}. Between 100 and 500 ms after cue onset, a target was turned on at the cued location (valid cue), the fixation point was simultaneously extinguished, and the monkeys then made a saccadic eye movement to fixate that target. On 20% of the experimental trials, the cue and target were in opposite visual fields (invalid cue).

In the symbolic cueing task, the monkeys fixated centrally, and a large, coloured cue overlapped the fixation point. The colour encoded the location where the monkey would make an eye movement. A red cue symbolized that the target would appear in the right hemifield; a green foveal cue symbolized a left target. Valid cues were presented on 80% of the trials. In manual versions of the cueing tasks, cues and targets were presented as before, and the monkey released a bar with the right hand after right targets and with the left hand after left targets. No eye movements were allowed. Targets were dim (6% brighter than background) and briefly flashed.

The superior colliculus was stimulated during fixations and at various times after cue onsets. Stimulus trains were of 40 ms duration, at 400 Hz, consisting of 0.1-ms pulses at twice threshold amplitude. To prevent the monkeys from adapting to stimulation, 20% of all trials were made without it. Visual target positions were selected to be roughly orthogonal to the stimulation-evoked saccades.

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K_vLQT1 and IsK (minK) proteins associate to form the I_{Ks} cardiac potassium current

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IN mammalian cardiac cells, a variety of transient or sustained K⁺ currents contribute to the repolarization of action potentials¹. There are two main components of the delayed-rectifier sustained K⁺ current, I_{Kr} (rapid) and I_{Ks} (slow)². I_{Kr} is the product of the gene *HERG*^{3,4}, which is altered in the long-QT syndrome, LQT2 (ref. 5). A channel with properties similar to those of the I_{Ks} channel is produced when the cardiac protein IsK is expressed in *Xenopus* oocytes⁶⁻⁸. However, it is a small protein with a very unusual structure for a cation channel⁹⁻¹⁵. The *LQT1* gene is another gene associated with the LQT syndrome, a disorder that causes sudden death from ventricular arrhythmias¹⁶. Here we report the cloning of the full-length mouse K_vLQT1 complementary DNA and show that K_vLQT1 associates with IsK to form the channel underlying the I_{Ks} cardiac current, which is a target of class-III anti-arrhythmic drugs and is involved in the LQT₁ syndrome.

The function of the *KVLQT1* gene is not known, although a partial amino-acid sequence indicates that it might encode a potassium channel¹⁶. The full-length K_vLQT1 cDNA was cloned from a mouse heart library. Its sequence revealed an open reading frame encoding a 604-amino-acid polypeptide with 90.5% identity with the human K_vLQT1 partial sequence (Fig. 1a). Hydrophobicity analysis predicts a classic voltage-dependent K⁺ channel (K_v) topology with six transmembrane segments (of the *Shaker* type) and a long unique carboxy-terminal cytoplasmic domain (Fig. 1b). The phylogenetic tree of representative members of the K_v-type K⁺ channels (Fig. 1c) indicates a very distant relationship between K_vLQT1 and all the other K⁺ channels.

In COS cells, K_vLQT1 expression induced a voltage-dependent, rapidly activating and slowly deactivating outward K⁺ current (Fig. 2a). Although these kinetic properties are similar to those of I_{Kr} in cardiac myocytes², the K_vLQT1-induced current has an almost linear current-voltage relationship (Fig. 2d), whereas I_{Kr} has an apparent inward rectification at positive potentials³. The properties of the K_vLQT1 current differ from all known K⁺ outward currents described in ventricular cells¹, including I_{Ks} which is characterized by slow activation kinetics. Although when expressed in *Xenopus* oocytes it produces slow K⁺ current, the IsK gene does not

lead to expression of K⁺-channel activity in a number of transfected mammalian cells, including COS cells¹³. The absence of expressed K⁺ current in COS cells transfected with IsK alone is shown in Fig. 2b. Cotransfection of IsK with K_vLQT1 leads to the appearance of a current with an amplitude at least twice the amplitude of the K_vLQT1 current alone (Fig. 3c). Moreover, in the presence of IsK, the activation kinetics of the K_vLQT1 channel are slowed down considerably and acquire the characteristic sigmoidal shape of the onset of activation of the cardiac I_{Ks} channel². The K_vLQT1 + IsK current fully resembles the I_{Ks} current, including the activation threshold potential that is shifted from -40 mV for K_vLQT1 to -20 mV for K_vLQT1 + IsK (Fig. 2d). This potential has a value very similar to that reported for I_{Ks} in both guinea-pig² and mouse ventricular myocytes¹⁷. The deactivation tail currents are not significantly modified by the coexpression of IsK.

Our COS cell study leads us to suggest that previous results showing that the complementary RNA for IsK produces slow K⁺ channel activity in *Xenopus* oocytes with properties resembling I_{Ks} (refs 6-8) are in fact due to IsK activation of an endogenous oocyte K⁺ channel similar to K_vLQT1. This conclusion is now strengthened by expression experiments shown in Fig. 3. K⁺ currents recorded in K_vLQT1-injected *Xenopus* oocytes (Fig. 3a) are very similar to those observed in COS cells. A rapid activation ($\tau_{act} = 144$ ms at +30 mV) is associated with a slow deactivation ($\tau_{deact} = 740$ ms at -60 mV). Conversely, as in COS cells, the coexpression of K_vLQT1 with IsK reconstitutes the I_{Ks} current with its unique slow activation kinetics and with a considerably larger amplitude than the K_vLQT1 current alone (Fig.

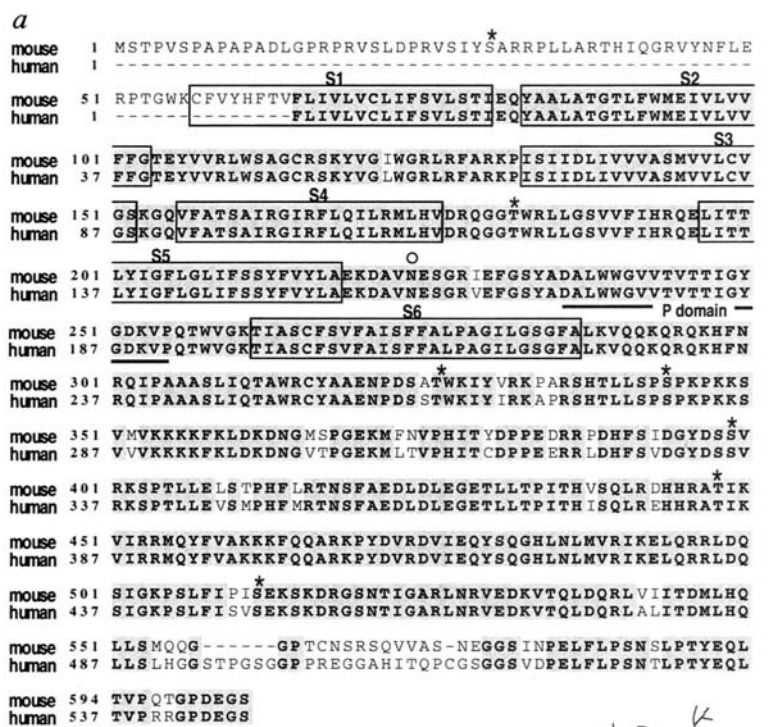


FIG. 1 a, Amino-acid sequence of mouse K_vLQT1 (Genbank accession number U70068) aligned with the human sequence. Identical amino acids are shown in bold, the six putative transmembrane segments, S1 to S6, are boxed, and the pore region (P domain) is underlined. (*) protein kinase C consensus phosphorylation sites; (O) potential glycosylation site. b, The molecular architectures of K_vLQT1 and IsK. c, Phylogenetic tree of the voltage-dependent K⁺ channels (generated using Genetic Computer Group software).

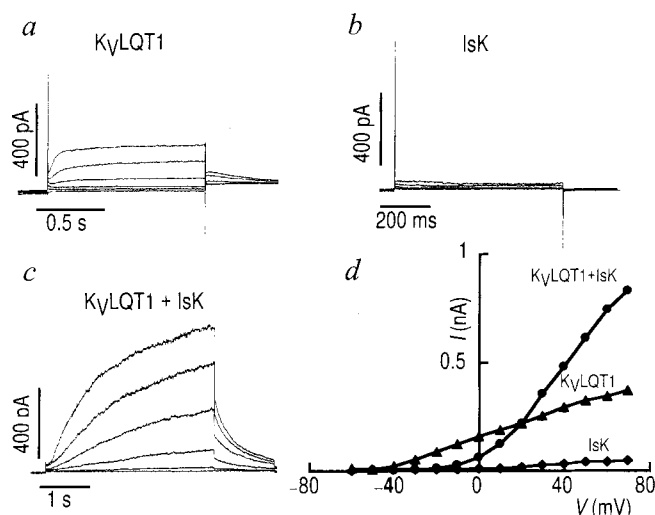


FIG. 2 Transient expression of IsK, K_vLQT1 and $K_vLQT1 + IsK$ in transfected COS cells. *a–c*, K^+ currents in response to voltage pulses from -60 mV to $+70$ mV in 20 -mV steps from a holding potential of -80 mV and repolarization to -40 mV. *a*, Expression of K_vLQT1 alone. *b*, Absence of expression of IsK³³ alone. *c*, Coexpression of $K_vLQT1 + IsK$. *d*, K^+ currents measured at the end of the test pulses are plotted as a function of membrane potential for IsK (diamonds), K_vLQT1 (triangles) and $K_vLQT1 + IsK$ (circles).

3b). The current elicited by IsK in *Xenopus* oocytes is also similar to I_{Ks} , but never reaches amplitude values higher than $\sim 1 \mu A$ irrespective of the amount of cRNA injected^{9,10,13}. In contrast, the amplitude of the $K_vLQT1 + IsK$ currents increases with increasing amounts of cRNA injected, reaching values as high as $20 \mu A$ (not shown). The reversal potential of tail currents varied with extracellular K^+ concentrations, with slopes of 52 mK (IsK, $n = 5$, $r = 0.994$), 52 mV (K_vLQT1 , $n = 6$, $r = 0.997$) and 51 mV ($K_vLQT1 + IsK$, $n = 5$, $r = 0.996$) per tenfold increase in K^+ concentration, indicating a good K^+ selectivity. Figure 3c, d compares the current-voltage relationships of the K_vLQT1 and $K_vLQT1 + IsK$ channels. In both cases, the currents are clearly outwardly rectifying, with activation thresholds of -53 ± 4 mV for K_vLQT1 and of -48 ± 3 mV for $K_vLQT1 + IsK$, with no decline for large depolarizations. For unknown reasons, the shift in the threshold of the activation potential induced by IsK is less pronounced in oocytes than in COS cells. The protein kinase C and Ca^{2+} regulation properties of both K_vLQT1 and $K_vLQT1 + IsK$ were found to be very similar to those of IsK alone¹⁸. Activation of protein kinase C by phorbol-12-myristate-13-acetate (PMA) decreased the currents (Fig. 4a, b), whereas raising the internal Ca^{2+} by inositol trisphosphate injection increased them (Fig. 4c, d).

Previous mutagenesis studies of IsK determined that the C-terminal domain of IsK was a critical structural element for the induction of K^+ currents^{9,19}. A mutation in this C-terminal domain, where threonine replaces serine at residue 68 (S68T) or its truncation at amino-acid 80 (Tr80), not only abolished the generation of K^+ currents, but also produced antagonists that inhibited the IsK-induced current in *Xenopus* oocytes. These results are in agreement with the view that IsK acts as a subsidiary subunit of an endogenous channel in oocytes. We have now observed that the S68T mutant of IsK has inhibitory effects on the $K_vLQT1 + IsK$ currents (Fig. 3e), as does IsKTr80 (not shown). Furthermore, the amino-terminal-deleted form, IsK $\Delta 11-38$, which can produce K^+ -channel activity by itself in oocytes, still has a strong stimulatory effect on K_vLQT1 activity. These findings indicate that IsK and its mutated derivatives exert the same action on the endogenous oocyte K^+ current and the cardiac channel subunit K_vLQT1 .

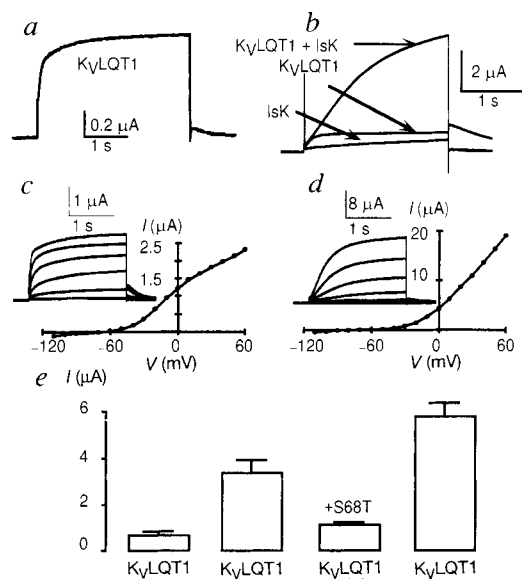


FIG. 3 Functional expression of K_vLQT1 and $K_vLQT1 + IsK$ in *Xenopus* oocytes. *a*, *b*, Mean current traces (depolarizing pulses to $+30$ mV from a holding potential of -60 mV) from 5 oocytes injected with 7 ng K_vLQT1 cRNA (*a*), or co-injected with K_vLQT1 (2 ng) + IsK (0.2 ng), or independently injected with the same quantities of K_vLQT1 and IsK³³ cRNAs (*b*). *c*, *d*, Current-voltage relationships of K_vLQT1 (7 ng) and K_vLQT1 (2 ng) + IsK (0.2 ng)-induced currents ($n = 10$). Insets, current traces induced by depolarizations from -60 mV to $+40$ mV in 20 -mV steps. *e*, Mean amplitudes of K^+ currents generated by injection of K_vLQT1 (7 ng) and by co-injection of K_vLQT1 (2 ng) + IsK (0.2 ng) and/or the indicated IsK mutants ($n = 10$).

Hybridization of RNA blots either with K_vLQT1 or IsK probes reveals a complete parallelism between the distribution of the two gene transcripts (not shown). The IsK and K_vLQT1 messenger RNAs are abundant in mouse heart, kidney and salivary glands, but are almost undetectable in brain, skeletal muscle and liver.

Immunoprecipitation experiments with the baculovirus-Sf9 system were used to demonstrate the formation of hetero-oligomeric complexes containing both K_vLQT1 and IsK. Sf9 cells were infected by recombinant FlagM2-tagged K_vLQT1 and native IsK baculoviruses, either independently or in combination. Electrophysiological recordings presented in Fig. 5a confirmed that these recombinant baculoviruses induce currents very similar to those induced by the corresponding pCI plasmids in COS cells. As

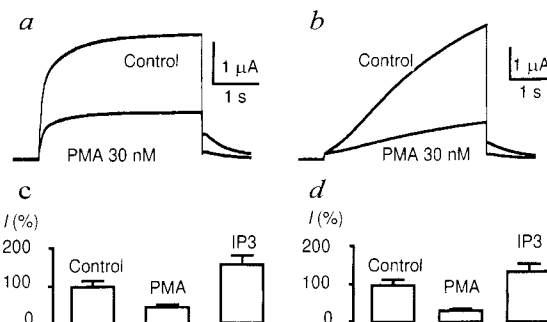


FIG. 4 Inhibition of K_vLQT1 (10 ng cRNA) (*a*) or $K_vLQT1 + IsK$ (0.2 ng + 0.02 cRNAs) (*b*) induced currents by application of 30 nM PMA during 30 min. Depolarizations to $+30$ mV. *c*, *d*, Effect of PMA (30 nM) and injection of inositol trisphosphate (IP3) on mean amplitudes of K_vLQT1 and $K_vLQT1 + IsK$ currents ($n = 10$).

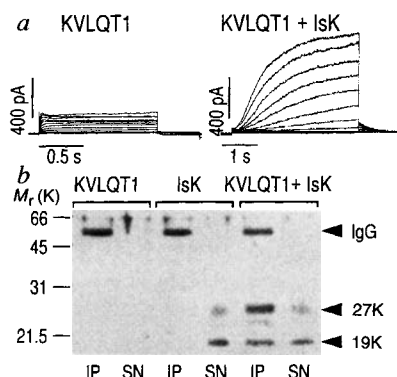


FIG. 5 *a*, K^+ currents expressed on Sf9 cells infected with FlagM2-tagged K_vLQT1 without (left) or with IsK^{13} recombinant baculoviruses. *b*, FlagM2-tagged K_vLQT1 and IsK proteins were coprecipitated by anti-FlagM2 antibodies from cells expressing both proteins (lane $K_vLQT1 + IsK$, IP), but not IsK alone (lane IsK , IP). A stronger association of the glycosylated form of IsK^{13} to K_vLQT1 is indicated by the enrichment of the $IsK27K$ (glycosylated) band in the precipitate as compared to the $19K$ (unglycosylated) band (last 2 lanes). The band at ~50K in IP lanes corresponds to the IgG heavy chains. IP, immunoprecipitate; SN, supernatant.

before, IsK did not produce any current when expressed alone, but dramatically increased the amplitude and slowed down the activation kinetics of the coexpressed K_vLQT1 current. The K_vLQT1 subunit was immunoprecipitated with anti-FlagM2 from solubilized Sf9 membrane, and the presence of IsK in the precipitated complex was determined by western blot using anti- IsK antibodies¹³. The results (Fig. 5b) clearly demonstrate the physical association of IsK and K_vLQT1 .

In conclusion, we have shown that the *KVLQT1* gene encodes the channel subunit forming the important cardiac channel I_{Ks} . However, this subunit is not sufficient by itself to form the native I_{Ks} , but requires IsK as an additional subunit. The I_{Kr} and I_{Ks} currents are the targets of anti-arrhythmic drugs and have an important impact on cardiovascular risk in controlling the ventricular repolarization process. With the recent identification of *HERG* as the gene encoding I_{Kr} (ref. 3), and the present demonstration that the association of K_vLQT1 and IsK reconstitutes I_{Ks} , both currents are now characterized at the molecular level. The link between the *KVLQT1* gene and I_{Ks} underlines the pathophysiological importance of this current in the human heart. The molecular identification of the I_{Ks} channel should help with the design of new anti-arrhythmic drugs. □

Methods

Cloning of K_vLQT1 cDNA. A 600-bp DNA fragment corresponding to the 5' end of the published human K_vLQT1 sequence¹⁶ was amplified using the polymerase chain reaction (PCR) from mouse heart poly(A)⁺-derived cDNA, subcloned into pBluescript (Stratagene), sequenced and used as a probe to screen 10⁶ recombinants of a mouse cardiac cDNA library²⁰. Two hybridization-positive clones were isolated and the cDNA plasmids were recovered by rescue excision. The DNA insert of one of them was fully sequenced on both strands (automatic Applied Biosystems sequencer, model 373A). The second DNA clone was sequenced on only one strand and found to be identical to the first clone. The coding sequence of K_vLQT1 was amplified using the low error rate PWO DNA polymerase (Boehringer) and subcloned into PEXO²¹ and pCI (Promega) plasmids for functional expression studies.

Electrophysiology. Methods of cRNA injection in *Xenopus* oocytes and electrophysiological recordings have been described²². The whole-cell configuration of the patch-clamp technique²³ was used to record K^+ currents on COS and Sf9 cells. The external solution at pH 7.4 contained (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES/NaOH. The pipette solution at pH 7.3 contained (in mM): 140 KCl, 2 MgCl₂, 10 HEPES/KOH, 2 EGTA.

Baculovirus expression. The 8-amino-acid FlagM2 epitope (Eastman Kodak) was added to the C terminus of K_vLQT1 by PCR techniques. The tagged coding sequence was subcloned into the transfer vector pVL392 and recombinant virus

prepared according to the manufacturer's instructions (Pharmingen). All the methods have been described²⁴.

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Coassembly of K_vLQT1 and minK (IsK) proteins to form cardiac I_{Ks} potassium channel

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THE slowly activating delayed-rectifier K^+ current, I_{Ks} , modulates the repolarization of cardiac action potentials. The molecular structure of the I_{Ks} channel is not known, but physiological data indicate that one component of the I_{Ks} channel is minK (refs 1–6), a 130-amino-acid protein with a single putative transmembrane domain⁷. The size and structure of this protein is such that it is unlikely that minK alone forms functional channels^{8,9}. We have previously used positional cloning techniques to define a new putative K^+ -channel gene, *KVLQT1*¹⁰. Mutations in this gene cause long-QT syndrome, an inherited disorder that increases the risk of sudden death from cardiac arrhythmias. Here we show that *KVLQT1* encodes a K^+ channel with biophysical properties unlike other known cardiac currents. We considered that K_vLQT1 might coassemble with another subunit to form functional channels in cardiac myocytes. Coexpression of K_vLQT1 with minK induced a current that was almost identical to cardiac I_{Ks} . Therefore, K_vLQT1 is the subunit that coassembles with minK to form I_{Ks} channels and I_{Ks} dysfunction is a cause of cardiac arrhythmia.

The previously described *KVLQT1* complementary DNA predicted a protein with six hydrophobic membrane-spanning α -helices (S1–S6), and a typical K^+ channel pore-signature

sequence¹¹. However, this cDNA seemed to be lacking the amino-terminal domain and was not able to undergo functional expression. To define the complete sequence of *KVLQT1*, we screened several cDNA libraries and isolated a new clone. This clone included a putative translational start site and an ATG sequence in-frame with the original *KVLQT1* clones. The new cDNA

sequence predicts a 581-amino-acid protein with a complete S1 domain and a 27-amino-acid N-terminal region (Fig. 1a). To ensure that this new sequence was part of the chromosome 11p15.5-linked *KVLQT1* gene, a 135-base pair (bp) *XhoI* restriction fragment from this region was used in hybridization experiments with DNA from a somatic cell hybrid panel. The new 5' end mapped to the short arm of chromosome 11. Northern analysis using the new *KVLQT1* sequence indicated hybridization with a single messenger RNA of 3.2 kb in human pancreas, heart, kidney, lung and placenta, but not in brain, liver or skeletal muscle (Fig. 1b).

To define the function of *K_vLQT1*, we transfected Chinese hamster ovary (CHO) cells with the new cDNA. A voltage-dependent, outward *K⁺* current was observed after membrane depolarization to potentials above -60 mV (Fig. 2a). This current reached a steady state within 1 s at +40 mV. Activation of the current was preceded by a brief delay, and repolarization to -70 mV elicited a tail current with an initial increase in amplitude (a hook) before deactivation. Similar tail current hooks were previously observed for *HERG K⁺* channels, and were attributed to recovery of channels from inactivation at a rate faster than deactivation¹²⁻¹⁴. The activation curve for *K_vLQT1* current was half-maximal ($V_{1/2}$) at -11.6 ± 0.6 mV, and had a slope factor of 12.6 ± 0.5 mV ($n = 6$; Fig. 2b).

The biophysical properties of *K_vLQT1* were unlike other

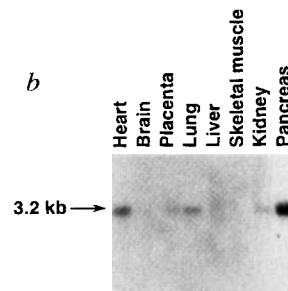
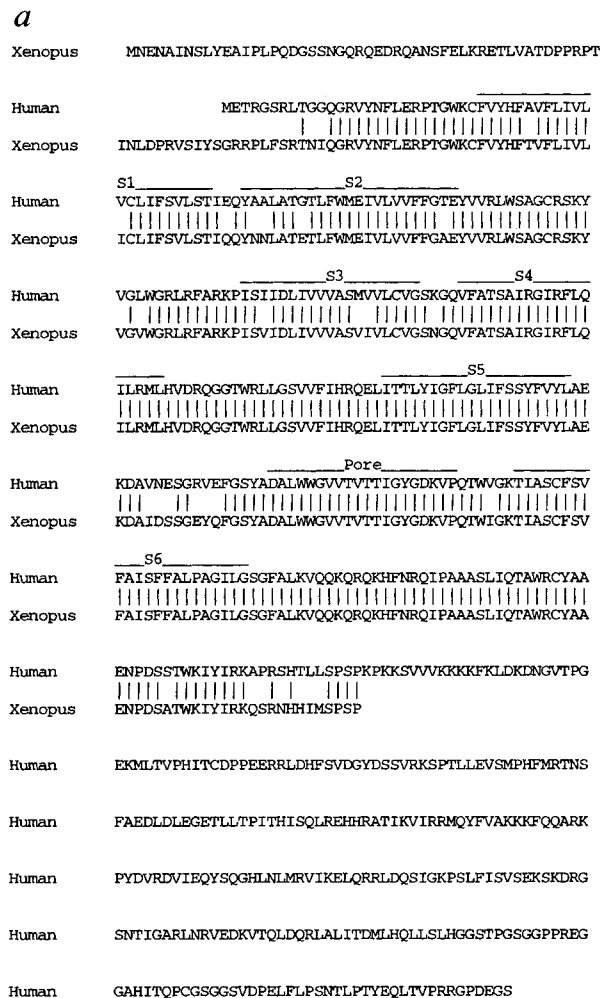


FIG. 1 Structure of human and *Xenopus K_vLQT1* and tissue-expression pattern of human *KVLQT1*. **a**, Comparison of human and a partial *Xenopus K_vLQT1* amino-acid sequence. Vertical lines indicate identical residues. **b**, Northern analysis showing expression of *KVLQT1* in human heart, placenta, lung, kidney and pancreas.

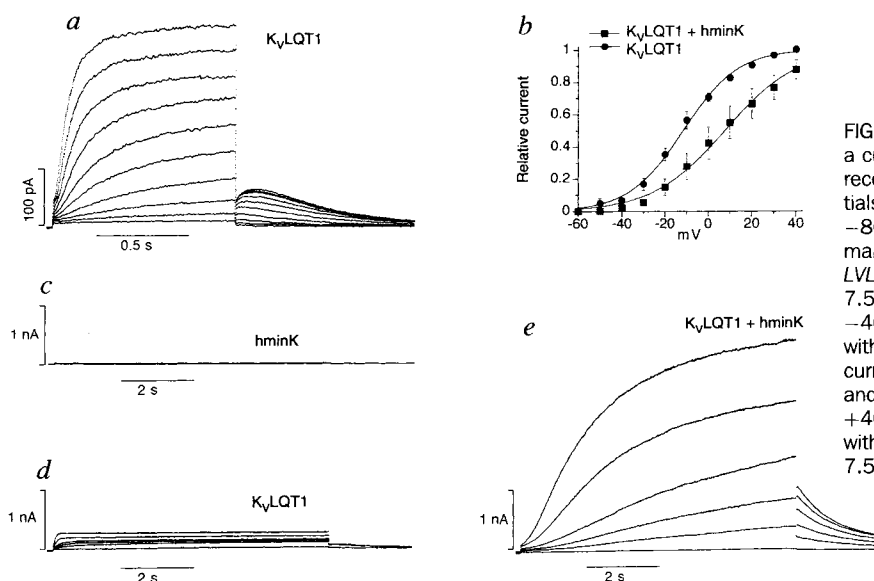
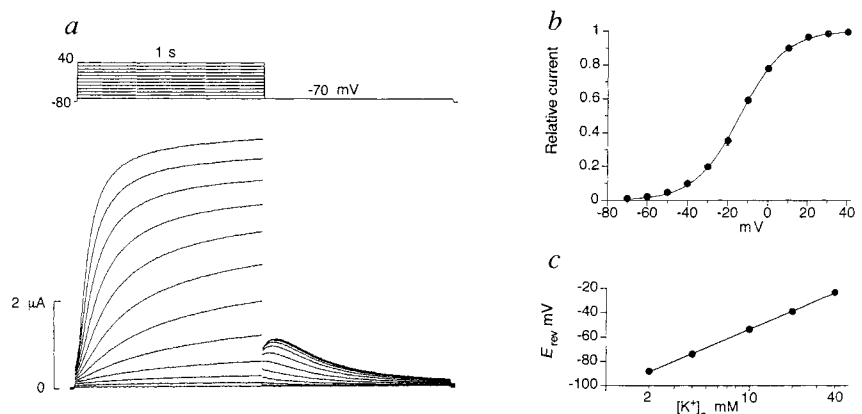


FIG. 2 *K_vLQT1* and *hminK* coexpression in CHO cells induces a current nearly identical to cardiac *I_{Ks}*. **a**, *K_vLQT1* currents recorded during 1-s depolarizing pulses to membrane potentials of -50 to +40 mV, applied from a holding potential of -80 mV. Tail currents were measured at -70 mV. **b**, Normalized isochronal activation curves for cells transfected with *LVLQT1* ($n = 6$; 1-s pulses) or *KVLQT1* and *hminK* ($n = 7$; 7.5-s pulses). **c-e**, Currents recorded during 7.5-s pulses to -40, -20, -10, 0, +20 and +40 mV in cells transfected with *hminK* (**c**), *KVLQT1* (**d**), or *KVLQT1* and *hminK* (**e**). Tail currents were measured at -70 mV in **d**, and at -50 mV in **c** and **e**. The amplitude of steady-state *K_vLQT1* current at +40 mV was 0.37 ± 0.14 nA ($n = 6$). In cells cotransfected with *KVLQT1* and *hminK*, time-dependent current during a 7.5-s pulse to +40 mV was 1.62 ± 0.39 nA ($n = 7$).

FIG. 3 Expression of K_VLQT1 in *Xenopus* oocytes. *a*, Currents recorded in an oocyte injected with 12.5 ng $KVLQT1$ cRNA. Pulses were applied in 10-mV increments from -70 to $+40$ mV. *b*, Isochronal (1 s) activation curve for K_VLQT1 current. The $V_{1/2}$ was -14.0 ± 0.2 mV and the slope factor was 11.2 ± 0.2 mV ($n = 9$). *c*, The relationship of E_{rev} versus $\log[K^+]_e$ was fitted with a linear function and had a slope of 49.9 ± 0.4 mV ($n = 6-7$ oocytes per point). Tail currents were measured at several voltages after 1.6-s prepulses to $+10$ mV.



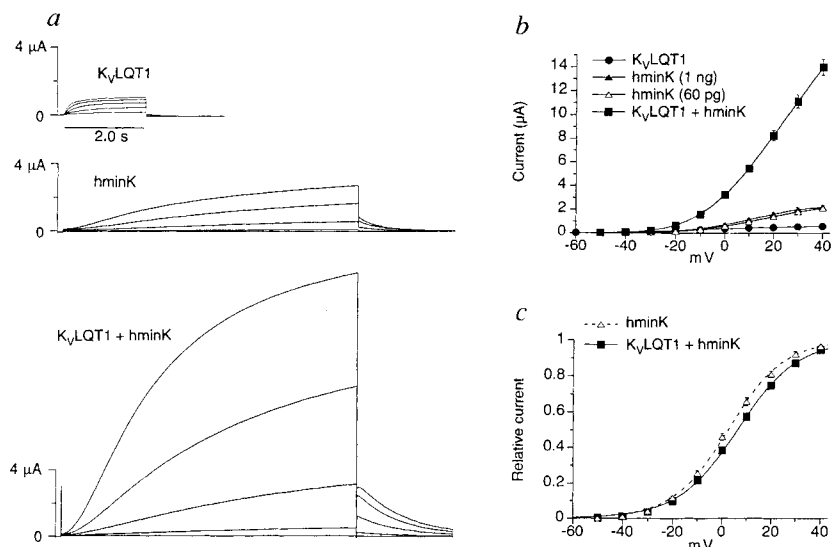
known cardiac K^+ currents. We considered that K_VLQT1 might coassemble with another subunit to form a known cardiac channel. To test this hypothesis, we cotransfected CHO cells with $KVLQT1$ and human *minK* (*hminK*) cDNAs. As reported previously⁹, transfection of CHO cells with *hminK* alone did not induce detectable current ($n = 10$, Fig. 2c). Cotransfection of *hminK* with $KVLQT1$ induced a slowly activating delayed-rectifier current that was much larger than the current in cells transfected with $KVLQT1$ alone (Fig. 2d, e). The slow activation of current in cotransfected CHO cells was preceded by a delay that lasted several hundred milliseconds, indicating that no significant homomeric K_VLQT1 channel current was present. Current did not saturate during long depolarizing pulses, and required a three-exponential function to best describe the initial delay and two phases of current activation. During a 30-s depolarizing pulse to $+40$ mV, current was activated with time constants of 0.68 ± 0.18 , 1.48 ± 0.16 , and 8.0 ± 0.6 s ($n = 4$). The isochronal (7.5 s) activation curve for current had a $V_{1/2}$ of 7.5 ± 0.9 mV, and a slope factor of 16.5 ± 0.8 mV ($n = 7$; Fig. 2b). By comparison, the $V_{1/2}$ and slope of the activation curve for human cardiac I_{Ks} are 9.4 mV and 11.8 mV¹⁵. Like K_VLQT1 and *hminK* coexpressed in CHO cells, activation of cardiac I_{Ks} is extremely slow and was best described by a three-exponential function^{16,17}. Quinidine (50 μ M) blocked tail currents in cotransfected CHO cells by $30 \pm 8\%$ ($n = 5$), similar to its effect (40–50% block) on I_{Ks} in isolated myocytes¹⁸. Thus, coexpression of K_VLQT1 and *hminK* in CHO cells induced a K^+ current with biophysical properties nearly identical to cardiac I_{Ks} .

To characterize the properties of *hminK* and K_VLQT1 further, we expressed these channels separately and together in *Xenopus*

oocytes. Oocytes injected with $KVLQT1$ complementary RNA expressed a rapidly activating outward K^+ current with a voltage dependence of activation nearly identical to CHO cells transfected with $KVLQT1$ cDNA (Fig. 3a, b). The K^+ selectivity of K_VLQT1 channels was determined by measuring the reversal potential (E_{rev}) of tail currents in different concentrations of extracellular K^+ ($[K^+]_e$). The slope of the relationship between E_{rev} and $\log[K^+]_e$ was 49.9 ± 0.4 mV ($n = 7$; Fig. 3c), significantly less than predicted by the Nernst equation (58 mV) for a perfectly selective K^+ channel. Co-injection of oocytes with $KVLQT1$ and *hminK* cRNA induced a current similar to I_{Ks} (Fig. 4a, bottom panel). The slope of the relationship between E_{rev} and $\log[K^+]_e$ for co-injected oocytes was 49.9 ± 4 mV ($n = 6$), similar to K_VLQT1 alone and to guinea-pig cardiac I_{Ks} (49 mV)¹⁹. The isochronal (7.5 s) activation curve for co-injected oocytes had a $V_{1/2}$ of 6.2 mV and a slope of 12.3 mV (Fig. 4c), similar to cardiac I_{Ks} .

By contrast with CHO cells, *hminK* was able to undergo functional expression in *Xenopus* oocytes (Fig. 4a, middle panel). The induced current (I_{SK}) was smaller than the current induced in co-injected oocytes, but the kinetics and voltage dependence of activation were similar (Fig. 4a–c). Two observations have led to the hypothesis that I_{SK} in *Xenopus* oocytes results from channels formed by coassembly of *minK* with an unidentified, constitutively expressed subunit. First, the magnitude of I_{SK} saturates after injection of very small amounts of *minK* cRNA (Fig. 4b), suggesting that an endogenous component of limited quantity is required for functional expression^{5,20}. Second, heterologous expression of *minK* in mammalian cells does not induce detectable current⁹ (Fig. 2c), suggesting that *minK* is not sufficient to form functional channels. We believed that this unidentified

FIG. 4 Coexpression of K_VLQT1 and *hminK* suggests that a K_VLQT1 homologue in *Xenopus* oocytes is present. *a*, Currents were recorded at -40 , -20 , 0 , $+20$ and $+40$ mV in oocytes injected with either 5.8 ng $KVLQT1$ (top), 1 ng *hminK* (middle), or co-injected with both cRNAs (bottom). *b*, Current–voltage relationships measured using 2-s pulses for K_VLQT1 , and 7.5-s pulses for *hminK*, or $KVLQT1$ and *hminK* ($n = 20$ cells for each condition). For oocytes injected with 60 pg or 1 ng of *hminK* cRNA, I_{SK} at $+40$ mV was 2.11 ± 0.12 μ A and 2.20 ± 0.18 μ A. *c*, Normalized isochronal activation curves for oocytes injected with *hminK* ($V_{1/2} = 2.4 \pm 0.3$ mV; slope = 11.4 ± 0.3 mV; $n = 16$) or co-injected with $KVLQT1$ and *hminK* cRNA ($V_{1/2} = 6.2 \pm 0.3$ mV; slope = 12.3 ± 0.2 mV; $n = 20$).



subunit might be a homologue of K_vLQT1 . To test this hypothesis, we screened a *Xenopus* oocyte cDNA library (Clontech) with a *KVLQT1* cDNA clone spanning the S3–S5 domains and isolated a 1.6-kb partial clone (*XKVLQT1*; Fig. 1a). XK_vLQT1 was 88% identical at the amino-acid level with the corresponding region of K_vLQT1 (Fig. 1a). These data suggest that I_{SK} results from the coassembly of the XK_vLQT1 and minK proteins.

We conclude that *KVLQT1* and minK coassemble to form the cardiac I_{Ks} channel. Two delayed-rectifier K^+ currents, I_{Kr} and I_{Ks} , modulate action-potential duration in cardiac myocytes^{15,17}. Our previous studies have implicated dysfunction of I_{Kr} channels in long-QT syndrome^{12,21,22}. The observation that *KVLQT1* mutations also cause this disorder¹⁰, and the discovery that K_vLQT1 forms part of the I_{Ks} channel, indicate that dysfunction of both cardiac delayed-rectifier K^+ channels contributes to the risk of sudden death from cardiac arrhythmia. □

Methods

Molecular cloning and northern analyses. A partial *KVLQT1* cDNA was radiolabelled with ³²P and used to screen several cDNA libraries (Clontech). A 1.2-kb clone was isolated from a pancreatic library and subcloned into pBlue-script II and sequenced¹⁰. Northern blots were analysed using a multiple-tissue northern filter (Human MTN blot 1; Clontech) as described²⁴.

Transfection and voltage clamp of CHO cells. *KVLQT1* and *hminK* cDNAs were subcloned into pCEP4 (Invitrogen). CHO cells were cultured in Ham's F-12 medium and transiently transfected using Lipofectamine (Gibco BRL). Cells were transfected for 18 h in 35-mm dishes containing 6 μ l lipofectamine, 0.5 μ g green fluorescent protein (pGreen Lantern-1; Gibco BRL), and 1.5 μ g of either *KVLQT1* or *hminK* cDNA in pCEP4. For cotransfection of *KVLQT1* and *hminK*, 0.75 μ g of each cDNA were used. Fluorescent cells were voltage-clamped using an Axopatch 200 patch clamp amplifier (Axon Instruments) 48 to 78 h after transfection. The bathing solution contained, in mM: 142 NaCl, 2 KCl, 1.2 MgCl₂, 1.8 CaCl₂, 11.1 glucose, 5.5 HEPES buffer (pH 7.4, 22–25 °C). The pipette solution contained, in mM: 110 potassium glutamate, 20 KCl, 1.0 MgCl₂, 5 EGTA, 5 K₂ATP, 10 HEPES (pH 7.3). Data acquisition and analyses were done using pCLAMP6 (Axon Instruments). The voltage dependence of current activation was determined by fitting the relationship between tail currents (determined by extrapolation of deactivating phase of current to the end of the test pulse) and test potential with a Boltzmann function. Tail currents were normalized relative to the largest value for each oocyte.

Voltage clamp of oocytes. *Xenopus laevis* oocytes were isolated and injected with cRNA as described¹². *KVLQT1* cDNA was subcloned into pSP64 (Promega). *HminK* cDNA was a gift from R. Swanson. Roughly equimolar concentrations of *KVLQT1* cRNA (5.8 ng per oocyte) and *hminK* (1 ng per oocyte) cRNA were used for the co-injection experiments. The bathing solution contained, in mM: 98 NaCl, 2 KCl, 2 MgCl₂, 0.1 CaCl₂, and 5 HEPES (pH 7.6, 22–25 °C). For reversal-potential experiments, osmolarity was maintained by equimolar substitution of external NaCl for KCl. Currents were recorded using standard two-microelectrode voltage clamp techniques 3 days after injection of oocytes with cRNA¹². Currents were filtered at 0.5 kHz and digitized at 2 kHz. Data are presented as mean $\pm$ s.e.m.

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Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides

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ENDOGENOUS neuromodulatory molecules are commonly coupled to specific metabolic enzymes to ensure rapid signal inactivation. Thus, acetylcholine is hydrolysed by acetylcholine esterase¹ and tryptamine neurotransmitters like serotonin are degraded by monoamine oxidases². Previously, we reported the structure and sleep-inducing properties of *cis*-9-octadecenamide, a lipid isolated from the cerebrospinal fluid of sleep-deprived cats³. *cis*-9-Octadecenamide, or oleamide, has since been shown to affect serotonergic systems⁴ and block gap-junction communication in glial cells (our unpublished results). We also identified a membrane-bound enzyme activity that hydrolyses oleamide to its inactive acid, oleic acid³. We now report the mechanism-based isolation, cloning and expression of this enzyme activity, originally named oleamide hydrolase⁵, from rat liver plasma membranes. We also show that oleamide hydrolase converts anandamide, a fatty-acid amide identified as the endogenous ligand for the cannabinoid receptor⁶, to arachidonic acid, indicating that oleamide hydrolase may serve as the general inactivating enzyme for a growing family of bioactive signalling molecules, the fatty-acid amides^{6–8}. Therefore we will hereafter refer to oleamide hydrolase as fatty-acid amide hydrolase, in recognition of the plurality of fatty-acid amides that the enzyme can accept as substrates.

Rat liver plasma membranes were isolated as described⁵ and solubilized in 10% glycerol, 1 mM EDTA, 20 mM HEPES, pH 7.2 (solubilization buffer) with 1% Triton X-100. From the detergent-solubilized fraction of the liver plasma membranes, oleamide hydrolase activity was partially purified by using a series of columns. After successive passage over DEAE, organomercurial, and heparin columns, only an approximately 20-fold enrichment of enzyme activity was achieved. Given this resistance of the enzyme to purification by conventional chromatographic techniques, we used a mechanism-based affinity chromatography approach based on the nanomolar inhibition of oleamide hydrolase activity by trifluoromethyl ketone **1** (Fig. 1a)⁵.

A linkable version of **1** (compound **4**) was synthesized (Fig. 1b) and attached by a disulphide bond to Sepharose beads. Upon passing the partially purified oleamide hydrolase activity over a column of **4**-derivatized beads, nearly all the oleamide hydrolase activity was removed from the flow-through solution (Fig. 1c). No detectable oleamide hydrolase activity was retained on a control column composed of beads derivatized with β -mercaptoethanol, indicating that the enzyme activity did not bind the bead matrix

itself. Elution of bound protein from the 4-derivatized column under reducing conditions released one major protein that migrated as a band corresponding to a relative molecular mass (M_r) of 60K–65K on SDS-PAGE (Fig. 1d, lane 7). This protein band was transferred to a PVDF (polyvinylidene difluoride) membrane, digested with trypsin, and seven of the resulting peptides were microsequenced. Degenerate oligonucleotides corresponding to the sequences of these peptides were then synthesized and used in the polymerase chain reaction (PCR) to clone a 350-base-pair (bp) complementary DNA fragment from rat liver messenger RNA. This partial clone was then used as a probe to screen a rat liver cDNA library for full-length clones. Isolation and fusion of two overlapping clones from the cDNA library afforded a putative full-length cDNA of 2,473 bases, which contains a single open reading frame encoding 63.3K of protein sequence (Fig. 2a).

Database searches for similarities with the fatty-acid amide hydrolase (FAAH) protein sequence identified extensive similarity to several amidase enzyme sequences from organisms as divergent as *Agrobacterium tumefaciens*⁹, *Pseudomonas savastanoi*¹⁰, *Aspergillus nidulans*¹¹, *Saccharomyces cerevisiae*¹², *Caenorhabditis elegans*¹³ and *Gallus domesticus*¹⁴. These amidases collectively make up an enzyme family¹⁵ whose members all share a common signature sequence (Fig. 2b). To our knowledge, FAAH is the first mammalian member of this family to have been molecularly characterized. A hydropathicity plot and transmembrane-domain searches (TMPred and PSORT programs) of the FAAH sequence revealed a strong putative transmembrane domain from amino acids 13–29 (bold in Fig. 2a). The 50-amino-acid region surrounding and encompassing the putative transmembrane domain of FAAH shares no homology with protein

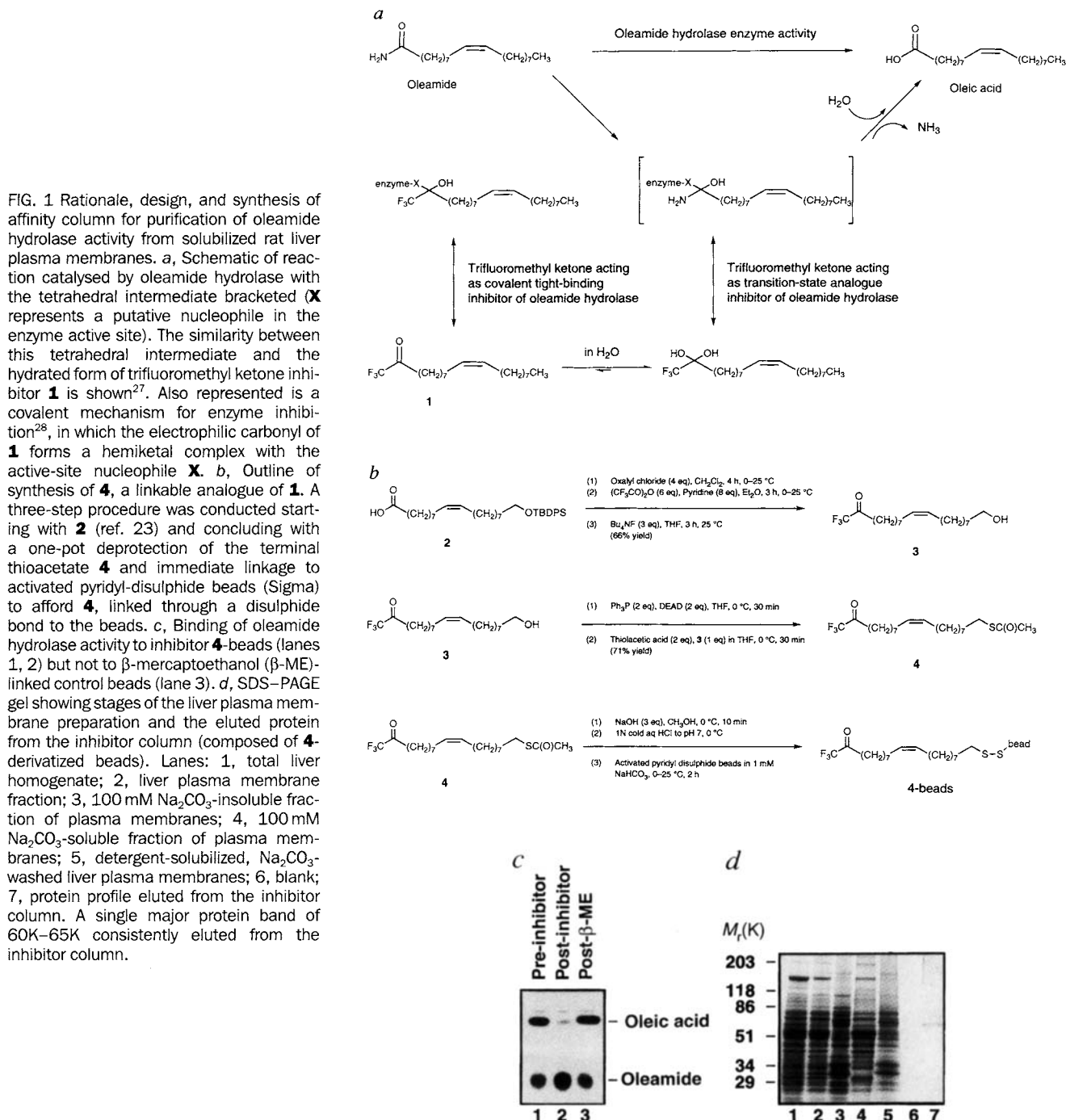


FIG. 1 Rationale, design, and synthesis of affinity column for purification of oleamide hydrolase activity from solubilized rat liver plasma membranes. **a**, Schematic of reaction catalysed by oleamide hydrolase with the tetrahedral intermediate bracketed (**X** represents a putative nucleophile in the enzyme active site). The similarity between this tetrahedral intermediate and the hydrated form of trifluoromethyl ketone inhibitor **1** is shown²⁷. Also represented is a covalent mechanism for enzyme inhibition²⁸, in which the electrophilic carbonyl of **1** forms a hemiketal complex with the active-site nucleophile **X**. **b**, Outline of synthesis of **4**, a linkable analogue of **1**. A three-step procedure was conducted starting with **2** (ref. 23) and concluding with a one-pot deprotection of the terminal thioacetate **4** and immediate linkage to activated pyridyl-disulphide beads (Sigma) to afford **4**, linked through a disulphide bond to the beads. **c**, Binding of oleamide hydrolase activity to inhibitor **4**-beads (lanes 1, 2) but not to β -mercaptoethanol (β -ME)-linked control beads (lane 3). **d**, SDS-PAGE gel showing stages of the liver plasma membrane preparation and the eluted protein from the inhibitor column (composed of **4**-derivatized beads). Lanes: 1, total liver homogenate; 2, liver plasma membrane fraction; 3, 100 mM Na_2CO_3 -insoluble fraction of plasma membranes; 4, 100 mM Na_2CO_3 -soluble fraction of plasma membranes; 5, detergent-solubilized, Na_2CO_3 -washed liver plasma membranes; 6, blank; 7, protein profile eluted from the inhibitor column. A single major protein band of 60K–65K consistently eluted from the inhibitor column.

sequences of other amidase family members, indicating that one of the unique modifications of the rat amidase may be its integration into the membrane. Additional analysis of the FAAH sequence revealed a polyproline segment (amino acids 307–315; double-underlined in Fig. 2a) which contains a precise match to the consensus class-II SH3-domain binding sequence, PPLPXR¹⁶, suggesting that other proteins may interact with FAAH to regulate its activity¹⁷ and/or subcellular localization¹⁸.

The FAAH cDNA was cloned into the eukaryotic expression vector pcDNA3 for transient expression studies in COS-7 cells. Whereas untransfected COS-7 cells contained negligible amounts of oleamide hydrolase activity, COS-7 cells transfected with the FAAH cDNA expressed high levels of oleamide hydrolase activity (Fig. 3a). This activity, like the rat liver plasma membrane oleamide hydrolase activity, was inhibited by trifluoromethyl ketone **1** (data not shown). Western blotting of the transfected COS-7 cell extract with polyclonal antibodies generated against an internal peptide sequence of FAAH revealed an immunoreactive band of *M_r* 60K–65K which comigrated with affinity-isolated FAAH on SDS–PAGE (Fig. 3b). Untransfected COS-7 cell extract contained no detectable immunoreactive protein band of this size. Also, the immunoreactivity of the 60K–65K protein was effectively competed out by preincubation of the antibodies with excess peptide antigen (Fig. 3b), whereas the trace quantities of cross-reactive protein present in transfected and untransfected COS-7 cell extracts did not compete with this peptide.

Previous work indicated that the enzyme that hydrolyses oleamide may be the same as that which converts anandamide (arachidonyl ethanolamide) to arachidonic acid^{19–22}. We therefore assayed COS-7 cells transfected with FAAH cDNA for anandamide hydrolase activity and found that whereas untransfected COS-7 cells had negligible anandamide hydrolase activity, this was

TABLE 1 Rates for FAAH-catalysed hydrolysis of fatty-acid amides

Substrate	Rate of hydrolysis	Per cent
Anandamide (100 μ M)	$333 \pm 30 \text{ nmol min}^{-1} \text{ mg}^{-1}$	100
Oleamide (100 μ M)	$242 \pm 20 \text{ nmol min}^{-1} \text{ mg}^{-1}$	72.6
Myristic amide (100 μ M)	$81 \pm 7 \text{ nmol min}^{-1} \text{ mg}^{-1}$	24.3
Palmitic amide (100 μ M)	$33 \pm 2 \text{ nmol min}^{-1} \text{ mg}^{-1}$	9.9
Oleamide (20 μ M)	$41 \pm 2 \text{ nmol min}^{-1} \text{ mg}^{-1}$	100
Stearic amide (20 μ M)	$2.3 \pm 1 \text{ nmol min}^{-1} \text{ mg}^{-1}$	5.8

Oleamide and anandamide hydrolysis rates are considered to be 100% of FAAH activity, to which other fatty-acid amide hydrolysis rates are compared.

high in transfected COS-7 cells (Fig. 3c). Thus, FAAH can hydrolyse both oleamide and anandamide, indicating that the amidase may act as a general degradative enzyme for the fatty-acid amide family of signalling molecules. Expressed FAAH could hydrolyse other fatty-acid amides, but at significantly reduced rates compared to oleamide and anandamide hydrolysis (Table 1). These data are consistent with previous work^{19–21}, in which the substrate selectivity of crude, tissue-derived FAAH was shown to be sensitive to both the chain length and degree of unsaturation of the substrate.

Southern and northern blots were analysed using an internal 800-bp fragment of the FAAH cDNA as a probe to evaluate the genomic copy number and tissue distribution of FAAH, respectively. Southern blots indicated that the FAAH probe hybridized primarily to single DNA fragments in several different restriction-enzyme digests of the rat genome (Fig. 4a); these results are consistent with the FAAH gene being a single-copy gene. North-

a

1-MVLSEVWTTLSGVSGVCLACSLLSAAVRLRWTRGRQKARGAATRAQRQKRA
51-SLETMDKAVQRFRLQNPDLSEALLTLPLQLVQKLQSGELSPEAVFFTY
101-LGKAWENVNGTNCVTSYLTDCETQLSQAPRQGLLYGVPVSLKECFYSYKGH
151-DSTLGLSLNEGMPSESDCVVQVLKLGAVPFVHTNVPQMSLSDCSNPL
201-FGQTMNPWKSSKSPGGSSGGEGALIGSGGSLGLGTDIGGSIRFSPAFCS
251-ICGLKPTGNRLSKSLGKCVYQGTAVQLSLGPMARDVESLALCLKALLCE
301-HLFTLDPTVPPLPFREEVYRSLRVRGVYETDNYTMPSPAMRALIETK
351-QRLEAAGHTLIPFLPNNIPYALEVLSAGGLFSDGGRSFLQNFKGDFVDPC
401-LGDLILILRLPSWFKRLLSLLKLPFLPRLAFLNSMRPSAEKLWLQHE
451-IEMYRQSVIAQWKAMNLDVLLTPMLGPALDLNTPGRATGAISYTVLYNCL
501-DFFAGVVPVTTVTAEDDAQMELYKGYFGDIWDIILKKAMKNSVGLPVAVQ
551-CVALPWQEEELCLRFMRVEQLMTPQKQPS-579

b

Fatty-acid amide hydrolase (rat)	215- GGSSGGEGALIGSGGSLGLGTDIGGSIRF PS-246
Propionamidase (chick)	222- GGSSGGEGALIAGGSSLLIGSDVAGSIR LPS-253
Putative amidase (<i>C. elegans</i>)	212- GGSSGGEGALIGAGGSLIGIGTDVGGSVRIP C-243
Putative amidase (<i>C. elegans</i>)	213- GGSSGGESALISADGSLLGIGDVGGSIRIP C-244
Putative amidase (<i>S. cerevisiae</i>)	207- GGSSGGEGSLIGAHSLLGLGTDIGGSIRIP S-238
Acetamidase (<i>Aspergillus</i>)	202- GGSSGGEGAIVGIRGGVIGVGTDIGGSIDVPA -233
Indoleacetamidase (<i>Agrobacterium</i>)	147- GGSSGGVAAAVASRLMLGGIGTDIGASVRLPA -178
Indoleacetamidase (<i>Pseudomonas</i>)	144- GGSSGGVAAAVASGIVPLSVGTDTGG SIRIPA-175

FIG. 2 Deduced amino-acid sequence of cloned FAAH and sequence comparison to other amidase family members. a, The deduced amino-acid sequence of FAAH. Bold type indicates the putative transmembrane-spanning domain as predicted by PSORT (psort.nibb.ac.jp) and TMPred (ulrec3.unil.ch/software/TMPRED_form.html) programs. Underlined sequence represents the seven peptide fragments from the trypsin-digested purified protein; double-underlined segment is the putative SH3-domain-binding sequence¹⁶. The FAAH cDNA GenBank accession number is U72497. b, Alignment over the amidase signature sequence region¹⁵ of the fatty-acid amide hydrolase with several other representative amidases. Residues of the signature sequence that are completely conserved among the amidase family members are shown in bold type and the relative amino-acid position of the signature sequence in each amidase is given by the numbers just preceding and following the sequence information.

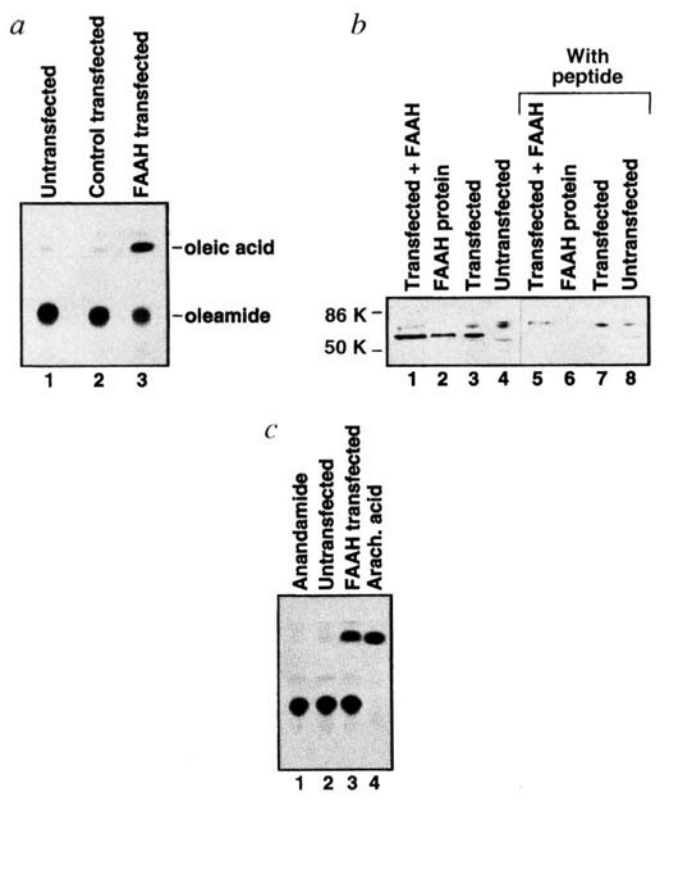


FIG. 4 Southern and northern blots probed with an internal 800-bp fragment of FAAH cDNA. *a*, Southern blot of rat genomic DNA digested with several restriction enzymes (10 µg of digested DNA per lane; restriction enzymes used were: lane 1, *EcoRI*; lane 2, *SacI*; lane 3, *BamHI*; lane 4, *HindIII*; lane 5, *XbaI*). *b*, Northern blot of rat mRNA from different rat tissues (2 µg mRNA).

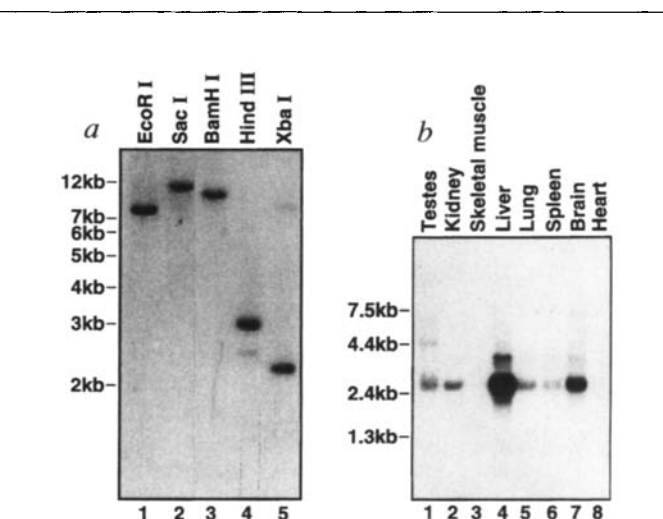
ern blots using the FAAH probe identified a single major mRNA transcript of ~2.5 kilobases (kb) that was most abundant in liver and brain, with lesser amounts in spleen, lung, kidney and testes (Fig. 4*b*). This transcript was not detectable in either heart or skeletal muscle, consistent with previous results that failed to identify any anandamide hydrolase activity in these tissues²². The northern blot also showed a small amount of hybridization of the FAAH cDNA probe to a few larger transcripts present only in those tissues expressing the 2.5-kb transcript as well. These transcripts may be either unprocessed or alternatively spliced forms of the 2.5-kb mRNA.

Given the activity described for different members of the fatty-acid amide family of signalling molecules⁶⁻⁸, the molecular characterization of a fatty-acid amide hydrolase reported here provides a framework for understanding the process that inactivates fatty-acid amides. The cloning of the gene encoding FAAH combined with the use of FAAH inhibitors⁵ should assist in determining which physiological pathways are affected by fatty-acid amides and how they might be intercepted pharmacologically. □

Methods

Preparation of 4-derivatized beads. Compound **2** (ref. 23) in CH₂Cl₂ (0.1 M) at 0 °C was treated with oxalyl chloride (4 equivalents) and stirred at 25 °C for 4 hours. Removal of solvent, followed by resolubilization in diethylether (0.1 M),

FIG. 3 Expression of FAAH in COS-7 cells. *a*, Thin-layer chromatography (TLC) demonstrating the conversion of ¹⁴C-oleamide to oleic acid in COS-7 cells transiently transfected with the FAAH cDNA in expression vector pcDNA3 (lane 3), but not in untransfected COS-7 cells (lane 1) or control transfected COS-7 cells (lane 2, transfected with pcDNA3 vector containing FAAH cDNA in reverse orientation). *b*, Western blot with rabbit polyclonal antibodies generated against an FAAH internal peptide sequence. Samples of untransfected COS-7 cell extract (lanes 4, 8), FAAH transfected COS-7 cell extract (lanes 3, 7), affinity-purified FAAH (lanes 2, 6), and a mixture of FAAH-transfected COS-7 cell extract and affinity-purified FAAH (lanes 1, 5) were probed with antibodies in the absence (lanes 1–4) or presence (lanes 5–8) of competing peptide antigen. FAAH-transfected COS-7 cell extract, but not untransfected COS-7 cell extract, contained an immunoreactive 60K–65K protein that co-migrated with purified FAAH. Only the immunoreactive 60K–65K protein can compete with excess peptide antigen, indicating that the other weakly immunoreactive protein bands present in both transfected and untransfected COS-7 cell extracts are the result of nonspecific antibody binding. *c*, Thin-layer chromatography demonstrating the conversion of ¹⁴C-anandamide to arachidonic acid in FAAH-transfected COS-7 cells (lane 3), but not in untransfected COS-7 cells (lane 2). TLC standards of anandamide and arachidonic acid are shown in lanes 1 and 4 respectively.



cooling to 0 °C, successive addition of trifluoroacetic anhydride (6 equivalents) and pyridine (8 equivalents), and stirring at 25 °C for 3 h yielded the crude silyl-protected trifluoromethyl keto alcohol upon aqueous work-up²⁴, which was deprotected without further purification by solubilization in tetrahydrofuran (THF; 0.1 M), addition of tetrabutylammonium fluoride (3 equivalents) and stirring at 25 °C for 3 h. Silica-gel column chromatography (gradient elution of 15–60% ethyl acetate–hexanes with 1% triethylamine) of the crude isolated product afforded **3** in 66% overall yield from **2**. To a solution of triphenylphosphine (2 equivalents) and diethyl azodicarboxylate (2 equivalents) in THF premixed at 0 °C for 30 min, thiolacetic acid (2 equivalents) and **3** (in THF to a final concentration of 0.1 M) were added successively and the reaction was stirred for 30 min at 0 °C. Work-up with saturated aqueous NaHCO₃ and silica-gel column chromatography (gradient elution of 0–20% ethyl acetate–hexanes with 1% triethylamine) gave **4** in 71% yield. Deprotection of **4** was accomplished with 1 M NaOH in methanol (3 equivalents) at 0 °C for 10 min, the reaction was neutralized with 1 M HCl to pH 7.0. Activated pyridyl disulphide beads (0.8 molar equivalents; Sigma) swollen in 1 mM NaHCO₃ were immediately added to the solution of deprotected **4** and the reaction shaken for 2 h at 25 °C. The **4**-derivatized beads were then washed with ethanol, followed by 1 mM NaHCO₃, and incubated with β-mercaptoethanol (3 equivalents) at pH 4.5 for 30 min at 25 °C to block any remaining activated thiol groups on the beads. The **4**-derivatized beads were then packed into a column, washed successively with ethanol and solubilization buffer with 0.2% Triton, and then treated with partially purified oleamide hydrolase activity as described below.

Purification of oleamide hydrolase activity. Oleamide hydrolase activity was partially purified by passing solubilized liver plasma membranes^{5,25} successively over columns of DEAE fast-flow Sepharose (Pharmacia; activity eluted with 50–200 mM NaCl; see ref. 3 for details of assay for oleamide hydrolase activity), organomercurial agarose (BioRad; activity eluted with 25 mM β-mercaptoethanol, 150 mM NaCl) and heparin–agarose (BioRad; oleamide hydrolase was

eluted using a 50 mM NaCl step gradient from 150–750 mM NaCl, with activity emerging in the 650–750 mM NaCl fractions). This procedure gave ~20-fold enrichment of oleamide hydrolase activity. The partially purified activity was subjected to a single gravity-flow passage over a 4-derivatized column (7 ml activity per ml column volume), and the column was washed with 20 volumes of solubilization buffer with 0.2% Triton X-100. The protein was eluted from the 4-derivatized column by passing 3 volumes of elution buffer (20 mM dithiothreitol (DTT) in solubilization buffer with 0.2% Triton X-100) over the inhibitor column and then incubating the column overnight at 4 °C. Subsequent elution with 1.5 volumes of elution buffer gave a single 60K–65K protein band (Fig. 1d, lane 7; 8–16% polyacrylamide gradient Tris-glycine gel (Novex)).

Cloning of FAAH cDNA. About 20 pmol of isolated 60K–65K protein was transferred to a PVDF membrane (BioRad) and digested *in situ* with trypsin²⁶. The resulting peptide fragments were eluted from the membrane, purified by high-performance liquid chromatography (HPLC), and HPLC fractions showing discrete peptide masses (as measured by matrix-assisted laser desorption/ionization with time of flight (MALDI-TOF; PerSeptive Biosystems linear instrument mass spectrometry) were microsequenced by automated Edman degradation chemistry (Applied Biosystems/Perkins Elmer procise 494 instrument). Degenerate oligonucleotides corresponding to portions of the sequenced peptides were synthesized and then used as primers in the polymerase chain reaction (PCR) with reverse-transcribed rat liver mRNA as template (PCR conditions: 94 °C, 30 s; 60 °C, 45 s; 72 °C, 60 s; 40 cycles). A 350-bp fragment of FAAH was isolated, cloned into pBluescript II SK(+) (Stratagene), internally radiolabelled with [α -³²P]CTP, and used as a probe to screen a rat liver λ gt11 cDNA library (Clontech). Four positive clones were identified from a screening of 4.5×10^5 plaques, and the two largest positive cDNA inserts were cloned into pBluescript II SK(–) and sequenced. Fusion of the two clones through an internal overlapping restriction site generated the putative full-length FAAH cDNA of 2,473 base pairs.

Expression of FAAH cDNA in COS-7 cells. The 2.47-kb FAAH cDNA was excised from pBluescript II SK(+) and ligated into the eukaryotic expression vector pcDNA3 (Invitrogen). 100-mm dishes of COS-7 cells were grown at 37 °C to 70% confluency in complete medium (DMEM with L-glutamine, non-essential amino acids, sodium pyruvate and FBS). The cells were then washed with serum-free medium and treated with 5 ml of transfection solution (5–6 μ g FAAH-pcDNA3 vector with transfectamine (Gibco-BRL)) and incubated at 37 °C for 5 h, at which point 10 ml of complete medium was added and incubation continued at 37 °C for 36 h, with one change of medium at 12 h. The COS-7 cells were collected, washed twice with 1 mM NaHCO₃, and Dounce-homogenized in 1 mM NaHCO₃. The resulting cell extract was used to assay for oleamide hydrolase³. Control COS-7 cells were prepared in the same way, except that the pcDNA3 vector used for transfection contained the FAAH cDNA in reverse orientation. Samples of cell extract from FAAH-transfected and untransfected COS-7 cells with about equal protein amounts were heated to 65 °C for 10 min in loading buffer with 2% SDS and 5% β -mercaptoethanol. Samples were then run on an 8–16% polyacrylamide gradient Tris-glycine gel and transferred to nitrocellulose for western blotting (using polyclonal antibodies at 15 μ g ml^{–1}). For peptide competition experiments, a 1,000-fold molar excess of peptide antigen was preincubated with polyclonal antibodies for 30 min before adding antibodies to the blot. ¹⁴C-fatty-acid amides were synthesized as described³, except that in the case of anandamide, instead of saturated aqueous NH₄OH, excess ethanolamine was added to the synthesized acid chloride. FAAH hydrolysis was assayed in duplicate with 100 μ M substrate (50 Ci mol^{–1}) and 3.5 μ g COS-7 cell protein extract for 5 min at 37 °C in 125 mM Tris-HCl, pH 9.0 (except in the case of stearamide, where owing to low solubility, 20 μ M substrate was used). Radio-labelled products were separated by TLC, eluted from silica with scintillation fluid, and counted. Substrate hydrolysis in the presence of equal amounts of untransfected COS-7 cell protein served as background control and was subtracted from transfected FAAH hydrolysis rates (Table 1).

Southern and northern analyses with FAAH cDNA. 10 μ g rat genomic DNA was digested with the indicated restriction enzymes (100 units each) for 12 h and then run on a 0.8% agarose gel. The DNA was then transferred under capillary pressure to a GeneScreenPlus hybridization transfer membrane (DuPont NEN) for Southern blotting. The northern blot (Clontech) was handled according to the manufacturer's guidelines, except that there was an additional post-hybridization wash (0.1% SDS, 0.1 $\times$ SSC, pH 7.0) at 65 °C for 1 h.

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A nucleotide-flipping mechanism from the structure of human uracil–DNA glycosylase bound to DNA

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ANY uracil bases in DNA, a result of either misincorporation or deamination of cytosine, are removed by uracil–DNA glycosylase (UDG), one of the most efficient and specific of the base-excision DNA-repair enzymes¹. Crystal structures of human^{2,3} and viral⁴ UDGs complexed with free uracil have indicated that the enzyme binds an extrahelical uracil. Such binding of undamaged extrahelical bases has been seen in the structures of two bacterial methyltransferases^{5,6} and bacteriophage T4 endonuclease V (ref. 7). Here we characterize the DNA binding and kinetics of several engineered human UDG mutants and present the crystal structure of one of these, which to our knowledge represents the first structure of any eukaryotic DNA repair enzyme in complex with its damaged, target DNA. Electrostatic orientation along the UDG active site, insertion of an amino acid (residue 272) into the DNA through the minor groove, and compression of the DNA backbone flanking the uracil all result in the flipping-out of the damaged base from the DNA major groove, allowing specific recognition of its phosphate, deoxyribose and uracil moieties. Our structure thus provides a view of a productive complex specific for cleavage of uracil from DNA and also reveals the basis for the enzyme-assisted nucleotide flipping by this critical DNA-repair enzyme.

UDG belongs to the base-excision repair pathway that repairs the more than 10,000 bases damaged daily in each human cell⁸. The most common forms of DNA damage occur by oxidation, depurination, alkylation and deamination. Cytosine deamination alone amounts to 100–500 uracil bases per cell per day¹. UDG

initiates base-excision repair by specifically recognizing and removing uracil from DNA. Subsequently, AP endonuclease cleaves the DNA backbone⁹, deoxyriphosphodiesterase removes the 5'-phosphate group, and DNA polymerase and DNA ligase replace the correct nucleotide¹. For UDG and

other enzymes, the pathway and mechanism leading to an extra-helical base remain mysterious: possibilities range from enzymatic capture of spontaneously flipped bases to enzyme-assisted flipping¹⁰. However, a conserved leucine residue at position 272, located above the UDG base-specificity pocket, was postulated

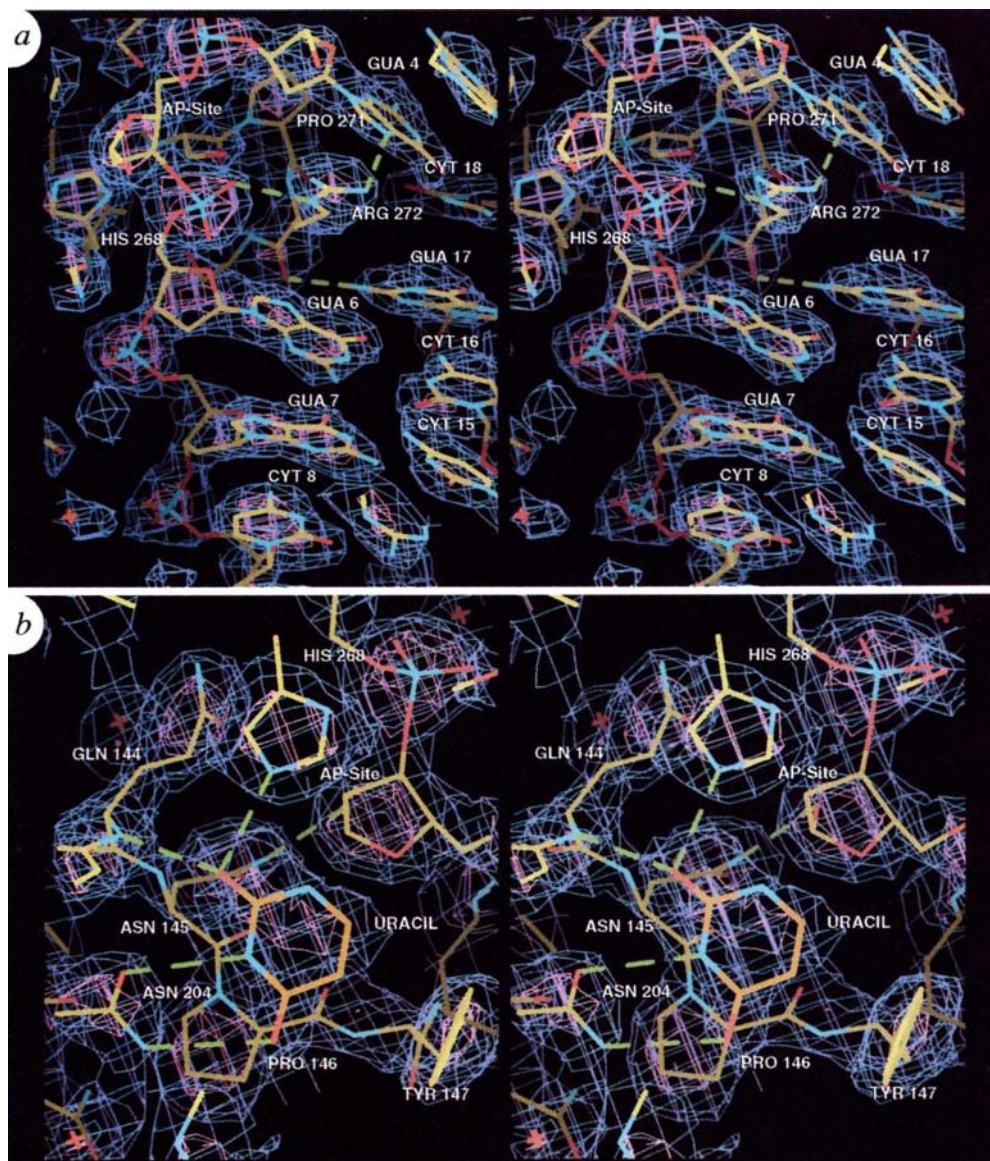
TABLE 1 Kinetics and DNA binding of UDG and mutant enzymes

		L272 (WT)	L272A	L272R	L272R/D145N
K_M (μ M)	dsDNA	0.8	3.8	0.3	0.7
	ssDNA	0.8	1.1	0.3	0.6
k_{cat} (min^{-1})	dsDNA	740	116	94	0.05
	ssDNA	1,178	261	136	0.13
k_{cat}/K_M ($\mu\text{M}^{-1}\text{min}^{-1}$)	dsDNA	925	31	303	0.07
	ssDNA	1,436	231	523	0.21
DNA binding*	dsDNA	56	28	173	160
	ssDNA	201	131	351	360

Kinetic experiments were done using seven different concentrations of [³H]dUMP-containing calf-thymus DNA and assayed as described¹¹. Individual kinetic parameters were calculated from direct linear plots using the Enzpack3 software package (Biosoft, UK). Standard errors are <10%.

* DNA-binding values represent NaCl concentration (mM) at peak elution from dsDNA–cellulose and ssDNA–agarose columns, respectively. Results are the mean of 3 independent experiments. Standard deviations are <10%.

FIG. 1 Stereo views of Arg 272 insertion into DNA and the bound flipped-out nucleotide. Electron density maps are contoured at 1.2σ (blue) and 3σ (pink) with blue phosphorus, red oxygen, blue nitrogen and yellow carbon bonds, together with selected hydrogen bonds (green dashed lines). **a**, Arg 272 insertion into DNA is viewed from the major groove, showing the σ_A -weighted $2F_o - F_c$ electron density map and DNA hydrogen-bond interactions with the Arg 272 backbone and side-chain atoms (Arg 272 NH_2 to phosphate G4 is not shown). Red crosses represent bound water molecules. **b**, Active site with bound DNA and uracil products viewed from the side of the uracil specificity pocket, and showing the σ_A -weighted $2F_o - F_c$ electron density map and hydrogen-bond interactions with uracil Watson–Crick atoms and the C1' hydroxyl of the abasic deoxyribose. Note that UDG L272R/D145N has cleaved the N–C1' glycosylic bond.



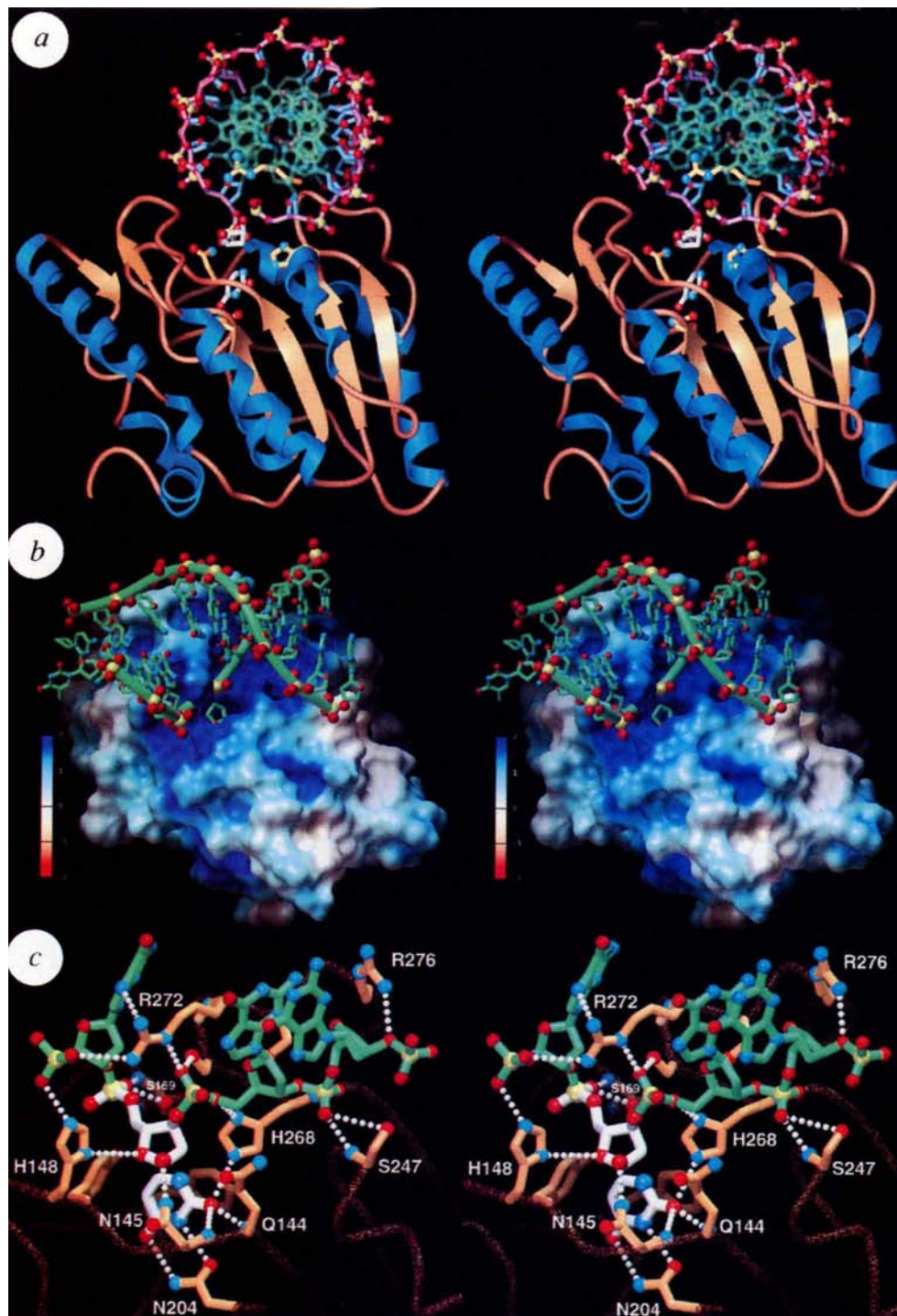
to play an active role in uracil recognition and base flipping². We designed active-site mutants of Leu 272 and the catalytically important residue Asp 145 to allow combined kinetic and structural studies aimed at understanding UDG's specificity and efficient activity.

The DNA-binding and kinetics for wild-type (L272) and three mutant UDGs (L272R, L272A and L272R/D145N) suggest that the side chain of residue 272 promotes uracil recognition and excision from DNA (Table 1). All three mutants have reduced k_{cat}/K_M . For L272A, enzyme activity and double-stranded (ds) DNA binding are severely impaired, with k_{cat}/K_M for single-stranded (ss) and dsDNA being only 16% and 3%, respectively, of that of L272 (Table 1), reflecting a sharply increased K_M for

dsDNA and strongly reduced activity. This reduced k_{cat} for L272A compared with that for L272 and L272R occurs even for uracil opposite an abasic site (data not shown) and for ssDNA (Table 1). Leucine 272 also contributes to DNA binding, as the overall DNA affinity of L272A is less than that of L272 (Table 1). For L272R, k_{cat}/K_M for ss- and dsDNA is 36% and 33% that of L272, respectively (Table 1). The decreased K_M of L272R indicates an enhanced DNA affinity which may slow the dissociation rate of the reaction products and lead to the observed decrease in k_{cat} .

We determined the structure of a UDG–DNA complex by combining the enhanced DNA binding of L272R with the reduced activity of a D145N mutant³ to create the double mutant L272R/D145N (Table 1), which was co-crystallized with a 10-base dsDNA

FIG. 2 Stereo views of the UDG–DNA complex. *a*, The complex is viewed from the 5' end of the uracil strand, showing its helical axis approximately perpendicular to the C-terminal edge of the four-stranded parallel UDG β -sheet (yellow arrows), with the residue-272 loop (orange) inserted into the minor groove. Arg 272 (yellow) occupies the abasic site within the DNA base stack (green) and protrudes into the major groove, stabilizing the extrahelical conformation of the flipped-out uracil nucleotide (white). Clockwise, from centre, UDG side chains Arg 272, His 268, Asn 204 and Asn 145 (yellow) surround the extrahelical nucleotide. *b*, Electrostatic orientation of UDG to bind DNA within the positively charged DNA-binding groove surface. The surface is coloured according to its electrostatic potential ($\leq 2kT/e$ to $\geq 2kT/e$) as illustrated in the left bar. The uracil-containing strand runs parallel with, and is buried in, the DNA-binding groove, with Arg 272 inserted into DNA and the 272 loop wedged into the minor groove. The abasic deoxyribose is at the top of the UDG specificity pocket directly below Arg 272. The uracil is buried deeply within the specificity pocket. The direction of the uracil-containing strand is 3' to 5' from left to right. *c*, Active-site interactions between the key nucleotides (green; G4, G6 and G7), the major UDG residues (orange) and the flipped-out nucleotide (white; U5). Hydrogen bonds (white dots) apparently stabilize and activate the flipped uracil nucleotide for the observed bond cleavage. Arg 272 (top centre) inserts between bases G4 and G6 above Ser 169 (back) and Ser 270, and adjacent to Ser 273 (clockwise right) behind the G6 ribose. Continuing clockwise, Arg 276, Ser 247 and His 268 form hydrogen bonds to phosphates, with the positively charged His 268 also forming a hydrogen bond to uracil O2. The uracil specificity pocket includes His 268, Gln 144, Phe 158 (behind), Asn 145, Asn 204 (bottom) and Tyr 147 (left). Above Tyr 147, positively charged His 148 forms hydrogen bonds to the abasic sugar and to the DNA phosphate 5' to G4.



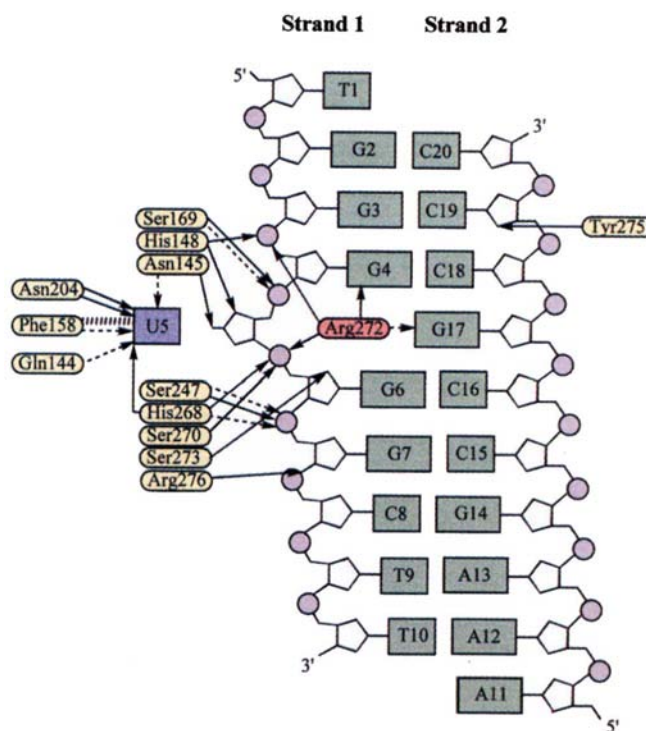


FIG. 3 UDg-DNA interactions. Nucleotide numbering follows the 5'-3' direction starting from the uracil-containing strand (strand 1). DNA bases are green, and phosphates are grey. Hydrogen-bond interactions with DNA are shown for amino-acid side chains (solid lines), and backbone atoms (dashed lines). Only one stacking interaction is shown for Phe 158 to uracil (thick broken line). Protein-DNA interactions are concentrated along the sugar-phosphate backbone of strand 1 near the flipped-out nucleotide.

oligonucleotide with a central U·G mismatch. The crystals belong to space group $P2_12_12_1$, $a = 48.8$, $b = 65.7$, $c = 97.1$ Å, and the structure was refined to an R value of 0.192 ($R_{\text{free}} = 0.282$) for data to 2.9 Å resolution. The well defined electron density reveals Arg 272 insertion into the DNA (Fig. 1a) and cleavage of the N-C1' glycosylic bond, with a deoxyribose C1' OH and free uracil bound in the specificity pocket (Fig. 1b). The structure is thus a trapped enzyme-product complex. The uracil ring is translated ~15 Å, and the uracil, deoxyribose and 5' phosphate are rotated 180° from their position in B-form DNA, hence the descriptive term nucleotide flipping. The bound DNA resembles B-form DNA 3' of uracil, with an average of 10.7 base pairs per turn and C2' endo

deoxyribose pseudorotation angles of 150° to 172°. The guanine 5' of uracil tilts towards the abasic step and its 3' phosphate moves towards the DNA-binding groove, with a local compression of the phosphate-phosphate distance flanking the flipped-out nucleotide (8.3 versus 11.5 Å for strand 2) and a ~20° shift in the helix axis.

The uracil-strand DNA backbone, which contributes 80% of the ~1,000 Å² of buried DNA surface area, binds along the UDg active-site groove (Fig. 2a), with the resulting UDg-DNA interface (~19 Å by ~23 Å) having extensive electrostatic complementarity (Fig. 2b). This interface is dominated by contributions from four successive nucleotides (63% of the buried DNA surface). The uracil 5' phosphate binds near an α -helix dipole and forms hydrogen bonds to Ser 169 NH and OH (Figs 2c, 3), whereas the G4 phosphate interacts with His 148. Downstream (3') of uracil, the Ser 270 and Ser 273 OH groups form hydrogen bonds with the G6 phosphate and O4', respectively. G7 O3' forms a hydrogen bond with Arg 276 NH₂, and the G7 5' phosphate, which is also stabilized by an α -helix dipole, interacts with Ser 247 NH and OH and His 268 NH (Fig. 3). The His 268 imidazole is within hydrogen-bonding distance of G6 O5' and uracil O2. These uracil-strand interactions are mainly responsible for UDg specificity as few interactions are seen with DNA strand 2 (Fig. 3). Above the UDg active site, the loop at position 272 penetrates the DNA minor groove and Arg 272 fills the abasic site and extends into the major groove. Arg 272 makes charged interactions to the G4 and G6 phosphates, its Ne forms a hydrogen bond to G4 N7, and its CO interacts with G17 N3 (Figs 1a, 3). These Arg 272-DNA interactions stabilize the extrahelical nucleotide conformation, which is consistent with the altered kinetic behaviour of L272R (Table 1).

The flipped-out nucleotide binds within the UDg specificity pocket, and contributes 24% of the buried DNA surface area. Specific interactions with the uracil and deoxyribose facilitate catalysis by polarizing the N-C1' bond and stabilizing the uracil and abasic sugar-leaving groups (Figs 1b, 2c). The deoxyribose forms hydrogen bonds from O4' and O1' to His 148 and Asn 145, respectively (Fig. 2c). His 148 also interacts with the G4 phosphate and thus delivers a charged hydrogen bond to O4'. The uracil stacks with Phe 158, and forms hydrogen bonds via its O4, N3 and O2 atoms to five residues: Phe 158 NH, Asn 204 side chain, the backbone atoms of residues 144 and 145, and the His 268 imidazole. His 268 also interacts with the uracil 3' phosphate (Fig. 2b), thus delivering a charged hydrogen bond to uracil O2 that can facilitate bond cleavage by stabilizing the developing oxyanion. Tyr 147 packs against uracil C5, excluding solvent and selecting against thymine. The mutation Y147A severely reduces k_{cat}^{11} , suggesting that bulk water exclusion is important for catalysis. In this buried active site, Asn 145 is rotated towards

FIG. 4 Concerted conformational changes that form the productive UDg-DNA complex are promoted by the process of assisting and stabilizing the flipped-out nucleotide. The DNA-bound UDg structure (orange) with uracil and the abasic sugar-phosphate (light grey) is superimposed upon free UDg (purple). Polar red oxygens, blue nitrogens and yellow phosphorus atoms are shown for key labelled UDg side chains and the flipped-out uracil nucleotide. The movements of Arg 272, His 148, Asn 145, and Asn 204 associated with DNA binding and nucleotide flipping promote concerted condensation around the bound uracil nucleotide which includes key catalytic residues His 268 and Asp 145 (Asn 145 in the current complex).



the bound uracil, where the wild-type Asp 145 may activate a water molecule, with the resultant hydroxyl nucleophile making a direct in-line attack on deoxyribose C1', as suggested previously⁴. Gln 144 and His 148 may orient the water molecule and shield the active site from bulk solvent. As DNA uracil is cleaved in our structure (Fig. 1b), the multiple interactions between UDG and the uracil deoxyribose evidently provide sufficient N–C1' bond polarization for cleavage, despite the debilitating D145N mutation.

Charge and shape complementarity between UDG and DNA (Fig. 2b) orients UDG for insertion of the side chain of residue 272 into the minor groove. Key enzyme conformational changes occur in the loops that interact with DNA, particularly the Leu 272 loop, which shifts towards the active site (Fig. 4). Productive binding involves full Leu 272 insertion, DNA backbone compression between flanking phosphates, and specific recognition of the flipped 5' phosphate, deoxyribose and uracil (Figs 2c, 3). Full penetration of side-chain 272 is accompanied by a nucleotide-expulsion ('push'), movement of the 5' phosphate towards Ser 169, and binding of Ser 270 and His 268 to the 3' phosphate, which acts as a pivot point for the dUMP. As uracil flips into the specificity pocket, numerous interactions with conserved UDG residues facilitate final productive binding ('pull'). His 268, linked to Leu 272 by the intervening conserved and rigid sequence Pro-Ser-Pro, moves ~2 Å to interact with uracil O2. The 272 loop movement is specific for productive uracil binding within DNA as it is absent in the UDG 6-aminouracil³, UDG-Ugi^{2,12} and viral UDG–nucleotide⁴ complexes, and free uracil is only an inhibitor at millimolar concentrations¹³. Thus UDG promotes uracil nucleotide substrate flipping and catalysis via a 'push–pull' mechanism involving DNA backbone compression, side-chain 272 penetration into DNA, and extrahelical nucleotide insertion into the recognition pocket. Together these UDG–DNA interactions result in concerted active-site shifts that form a productive complex (Fig. 4).

Such enzyme-assisted nucleotide flipping and coupled active-site interactions are supported by biochemical results. Abasic sites, which would facilitate 272 side-chain insertion, are competitive inhibitors of UDG at μM concentrations¹⁴. RNA uracil is not cleaved as the ribose 2'-OH and 3'-endo pucker would block His 268 movement. Uracil at the 3' end of dsDNA is repaired at a vastly reduced rate^{15,16} and the minimum substrate for uracil excision¹⁷ is p(dU)p(dN)p. Whereas mutations of Ser 270 and His 268 impair both UDG activity and DNA binding, mutation of Ser 169 only impairs activity³, suggesting that Ser 169 binding of the uracil 5' phosphate aids nucleotide flipping and catalysis. As a 5'-U is removed from ssDNA only when a 5' phosphate is present¹⁷, this 5' phosphate is evidently required for productive binding, as implied by the complex structure. Finally, uracil nucleotide analogues with altered deoxyribose configurations allow UDG binding but impair uracil excision¹⁸.

In the UDG–DNA product complex, the abasic sugar is displaced ~180° from its normal position, and this shift is mainly accommodated by unusual ζ and α torsion angles of its flanking phosphates (Fig. 5a). The 5' phosphate has torsion angles -sc for ζ and +ap for α , whereas the corresponding 3' torsion angles are -sc and -ac, none of which are normally seen in B-form DNA. However, this overall conformation of the flipped-out nucleotide is similar to the methyltransferase–DNA complexes^{5,6} in which the deoxycytidine and flanking phosphates are also rotated out of the dsDNA helix (Fig. 5b). Although enzymatic capture of spontaneously flipped bases could account for the slow catalysis of the methyltransferases ($k_{\text{cat}} \sim 0.03 \text{ min}^{-1}$)¹⁰, the 20,000 times increased rate of UDG ($k_{\text{cat}} = 740 \text{ min}^{-1}$; Table 1) and the UDG–DNA complex structure suggest that UDG catalyses nucleotide flipping, and that the methyltransferases may act by distinct but related mechanisms. The methyltransferases interact with DNA through the major groove, with the target nucleotide flipping out of the minor groove. In contrast, UDG mostly contacts the DNA minor groove, and the uracil nucleotide flips out of the major groove (Fig. 5). Structurally, the minor groove is

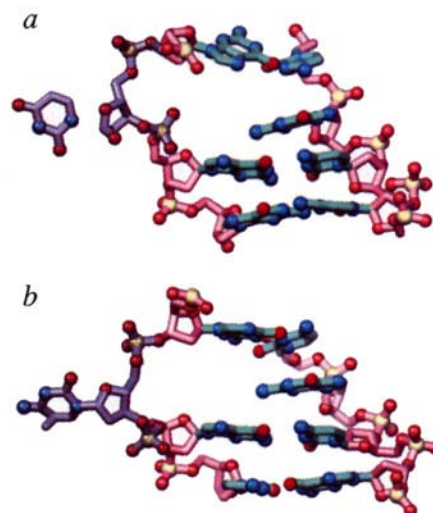


FIG. 5 Core DNA structures from the a, UDG, and b, *HhaI* cytosine methyltransferase⁵ enzyme–DNA complex crystal structures, showing their sugar–phosphate backbone distortions at the flipped-out nucleotides, including 5' and 3' phosphates (grey). The 5' phosphate, deoxyribose and base orientations are similar, despite uracil flipping out through the DNA major groove and cytosine through the DNA minor groove, which supports a related nucleotide-flipping mechanism. The flipped-base-containing strands run in the 5'–3' direction from the top. The DNA bases (green) and deoxyriboses are shown with the flipped-out base and sugar–phosphate (grey), together with the sugar–phosphate backbones of the four base pairs (pink) that dominate the UDG–DNA interface. Polar oxygen (red), nitrogen (blue) and phosphorus (yellow) atoms are shown as spheres.

less variable than the major groove, so abnormal base stacking and U–G wobble mispairs may be more easily probed from the minor groove, suggesting that minor groove interactions may be generally important for damaged base and mismatch detection. However, the similar DNA backbone recognition by UDG and by the methyltransferases, including the unusual flanking-phosphate torsion angles and backbone compression at the flipped nucleotide, suggests related mechanisms of enzyme-assisted nucleotide flipping.

Together, these results on the UDG–DNA complex provide a structural and biochemical characterization of nucleotide flipping by a eukaryotic DNA glycosylase. They establish an elegant and highly efficient mode of damaged base recognition and catalysis within dsDNA by a human DNA base-excision repair enzyme involving five principal components: (1) electrostatic orientation of the UDG active site along DNA; (2) damage detection and intercalation of Leu 272 via the DNA minor groove, promoting nucleotide flipping via the major groove and concerted UDG loop movements to form the productive complex; (3) binding to sugar–phosphates, promoting DNA backbone compression to favour the extrahelical nucleotide; (4) recognition of the flipped-out nucleotide base, deoxyribose and phosphate by the substrate-specificity pocket; and (5) uracil–deoxyribose N–C1' bond polarization by active-site hydrogen bonds. These features explain UDG's ability to recognize uracil in ss- and dsDNA and within different DNA sequence contexts. The extensive UDG–DNA backbone interactions revealed by this structure suggest that the backbone compression and 180° phosphate rotation promote base flipping by a nucleotide flipping mechanism. The versatility of nucleotide flipping as described here, and its likely conservation among eukaryotic and prokaryotic UDGs, indicate that this may be a widespread mechanism in DNA-base repair and modification processes, support its early evolution, and suggest that nucleotide flipping could emerge as a major mechanism for enzyme recognition of nucleotides within nucleic acid polymers. □

Methods

Mutagenesis and biochemistry. Site-directed mutagenesis, expression and purification of L272, L272R, L272A and L272R/D145N mutants have been described^{3,13}. UDG activity was measured in 20 μ l of assay buffer (10 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.5 mg ml⁻¹ BSA and 1.7 μ M [³H]dUMP-containing calf-thymus DNA)¹⁵, and the [³H]uracil released was measured¹⁵. For analysis of DNA-binding capacity, 10 μ g UDG in 500 μ l binding buffer (20 mM Tris-acetate, pH 7.0, 2 mM MgCl₂, 0.02% sodium azide, 10% glycerol (v/v) was loaded onto either ssDNA-agarose or dsDNA-cellulose (Pharmacia) columns (0.5 $\times$ 1.5 cm). Flow was maintained at 75 μ l min⁻¹ using the Pharmacia SMART system equipped with a conductivity monitor and an absorbance monitor simultaneously measuring absorbances at 260, 280 and 320 nm. Bound enzyme was eluted with a 3.6-ml linear gradient of NaCl (0–0.4 M) in binding buffer and the conductivity at peak elution was recorded.

Crystallization and data collection. Crystals were grown at 22 °C by sitting-drop vapour diffusion with equal volumes of UDG–DNA solution mixed with reservoir solutions of 20% polyethylene glycol 4000 (Sigma), 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) buffer (pH 6.5), 1 mM DTT. Crystals grow to full size in ~3 days, and contain one UDG–DNA complex per asymmetric unit. X-ray diffraction data were collected from a single crystal (0.5 $\times$ 0.2 $\times$ 0.06 mm, flash-frozen at -170 °C) at a wavelength of 1.08 Å on beamline 7-1 of the Stanford Synchrotron Radiation Laboratory. Data were collected using a 30-cm MAR research imaging plate and processed with DENZO and SCALEPACK¹⁹ yielding 21,922 observations of 7,140 unique reflections to 2.9 Å resolution (97% complete), with an average I/σ of 7.9 and an overall R_{sym} of 0.108, where R_{sym} is the unweighted R value on I between symmetry-related reflections.

Structure solution and refinement. The structure was solved by molecular replacement with AMoRe²⁰ using free UDG³ as a search model and all data from 4.0 to 10.0 Å resolution. The correct solution gave a correlation of 38% and an R value of 45%, with the corresponding values for the next highest peak 17% and 52%. This model was refined with X-PLOR 3.1 (ref. 21) and electron density for the DNA was clearly visible in σ_A -weighted²² $2F_o - F_c$, $F_o - F_c$ and simulated annealed omit electron density maps. Cycles of model building and refinement allowed all of the DNA to be placed. This complete model was refined using stereochemically restrained simulated annealing and positional refinement in X-PLOR. Manual inspection and rebuilding of the structure, as well as Fig. 1, was done with the interactive graphics program XFIT²³. Ordered solvent molecules were manually placed at the appropriate positions of $F_o - F_c$ electron density maps. Individual atomic temperature values were applied to all atoms and an overall anisotropic temperature factor correction applied to the native data set in the latter stages of the refinement. Progress was monitored throughout by the consistent decrease of the free R value. The human UDG–DNA complex structure contains 2,322 non-hydrogen protein atoms and 106 ordered solvent molecules and has been refined to an R value of 0.192 ($R_{\text{free}} = 0.282$) for 5,878 reflections in the resolution range 8.0 to 2.9 Å with $F > 3.0\sigma F$ (80% complete). The r.m.s. deviations from ideality²⁴ are 0.008 Å for bond lengths, 1.47° for bond

angles, 23.1° for dihedral angles, and 1.41° for improper angles. The average temperature values are 6.9 Å² for 893 protein main-chain atoms, 8.9 Å² for 960 protein side-chain atoms, 23.2 Å² for 405 DNA atoms and 16.3 Å² for 106 solvent molecules.

Structure analysis. Surface potential was calculated with version 2.1 of the University of Houston Brownian Dynamics program²⁵ using AMBER partial charges at pH 7, ionic strength of 150 mM and a radius of 1.6 Å for the solvent with the solute and solvent dielectric constants set to 2.0 and 78.0, respectively. Computer graphics analysis and rendering for Figs 2–5 were done using standard and local modules of the AVS graphics system (AVS, Waltham, MA). Buried surfaces were calculated using a 1.6 Å probe radius with the programs AREAIMOL and DIFFAREA of the CCP4 program suite (SERC (UK) Collaborative Computing Project No. 4, a Suite of Programs for Protein Crystallography, distributed from Daresbury Laboratory, Warrington WA4 4AD, UK).

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